TÉCNICO LISBOA

Study of Liquid Hot Water Fractioning Treatment using Wheat Straw Residues as Raw material in a Biorefinery Concept

Sugar Production for Biorefineries: Influence of Liquid Hot Water on Sugars, Lignin and Degradation Products using Wheat Straw

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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Declaration of Honour

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Institute of Chemical, Environmental and Bioscience Engineering of Technische Universität Wien (TU Wien) Vienna, Austria, during the period February-August 2020, under the supervision of Univ. Prof. Dipl. Eng. Dr.techn Anton Friedl and Univ. Ass. MSc. Eng. Sebastián Serna Loaiza, and within the frame of the PhD program TU Wien Bioactive - Technologies for Drug Discovery and Production. The thesis was co-supervised at Instituto Superior Técnico - Universidade de Lisboa (IST-UL) by Prof. Dr. Carla da Conceição Caramujo Rocha de Carvalho.

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To each and every one of you – Thank you so much for your friendship and love.

Resumo

O uso de material lignocelulósico tem sido estudado no contexto de biorefinarias. Contudo, o fracionamento de resíduos agro-industriais, como a palha de trigo, apresenta desafios devido à sua resistência à solubilização. Os processos de pré-tratamento têm como objetivo ultrapassar a barreira recalcitrante inerente à biomassa submetendo-a a processos fisico-químicos que facilitam a extração dos açúcares e de lignina. A inerente produção de substâncias inibidoras de processos fermentativos deve ser minimizada. Nesta tese, foi utilizado o método de autohidrólise isotérmica (com uma razão sólido-líquido de 1:11) a temperaturas de 160, 180 e 200˚C e tempos de espera de 30, 60 e 90 minutos. Foram também efetuados os balanços de massa de todos os passos de extração de modo a avaliar as perdas entre eles. Foram avaliados os perfis de concentrações de lignina, açúcares e produtos de degradação, na fração líquida, para cada uma das condições experimentais. A condição que produziu maior concentração de monómeros de açúcares (3,4 $\pm$ 0,05 g/L) foi de 180˚C durante 90 min no entanto, a que produziu mais açúcares oligoméricos (11,0 $\pm$ 0,5 g/L) foi a de 160˚C durante 90 min. Esta é também a condição máxima de temperatura e tempo de espera que pode ser imposta sem ultrapassar o limite de concentração de produtos de degradação. Hidrolisando os oligoaçúcares, esta concentração de açúcares totais pode chegar a 12,5 $\pm$ 0,2 g/L, a 180˚C durante 60 min. A condição experimental que satisfaz os critérios de produção de açúcares hemicelulósicos, limites de produtos tóxicos e de manutenção, quanto possível, da celulose e da lignina na fração sólida, para futura valorização, é 160˚C durante 90 min.

Palavras Chave

Água quente líquida; Açucares; Biorefinaria; Factor de serveridade; Lignina; Palha de trigo; Produtos de degradação; Pré-tratamento; Sustentabilidade.

Abstract

The use of lignocellulosic material has been studied in the biorefineries context. However, the fractioning of agro-industrial residues, such as wheat straw, presents processing challenges due to its solubilization resistance. The pretreatment processes aim to overcome the inherent recalcitrant barrier by subjecting the biomass to physical-chemical processes and facilitating the extraction of sugars and lignin. The inherent production of inhibiting substances of fermentative processes needs to be minimized. In this thesis, the method of isothermal autohydrolysis (with a solid-liquid ratio of 1:11) was used at temperatures of 160, 180 and 200˚C and holding times of 30, 60 and 90 minutes. Mass balances of all extraction steps were also performed in order to evaluate the losses between them. The lignin, sugars and degradation products profile in the liquid fraction were evaluated for each of the experimental conditions. The condition that produced the highest concentration of monomeric sugars (3.4 $\pm$ 0.05 g/L) was 180˚C for 90 min. However, the one that produced the most oligomeric sugars (11.0 $\pm$ 0.5 g/L) was 160˚C for 90 min. This is also the highest temperature and holding time that can be imposed by without surpassing the degradation products concentration threshold. Hydrolyzing the oligosugars, this concentration of total sugars can reach 12.5 $\pm$ 0.2 g/L, 180˚C for 60 min. The condition satisfies the criterium for the production of hemicellulosic sugars, limits of toxic products and maintaining both cellulose and lignin in the solid fraction of the process for future valorization is 160˚C for 90 min.

Keywords

Biorefinery; Degradation products; Lignin; Liquid hot water; Pretreatment; Severity factor; Sugars; Sustainability; Wheat straw

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Acronyms

1GBF	1st Generation Biofuels
2GBF	2nd Generation Biofuels
1GSP	1st Generation Sugar Production
2GSP	2nd Generation Sugar Production
ASL	Acid Soluble Lignin
AIL	Acid Insoluble Lignin
C5	Pentoses
C6	Hexoses
cGMP	Current Good Manufacture Practices
DP	Degree of Polymerization
DSP	Downstream Processing
HMF	5-hydroxymethylfurfural
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
IEA	International Energy Agency
LCB	Lignocellulosic Biomass
LHW	Liquid Hot Water
LSR	Liquid-to-solid Ratio
NTP	Normal Temperature and Pressure
R_0	Severity Factor
STR	Stirred Tank Reactor
SSS	Sugar Standard Solutions

SRS Sugar Recovery Standard

USP Upstream Processing

1

Introduction

Contents

1.1 General context and motivation

In the age of global warming and overconsumption - which are both a result of high worldwide resources demand and population growth - as well as the increase of the subsequent generated wastes (solid, liquid and gaseous) are damaging biodiversity all around the world by raising pollution levels in soil, oceans and atmosphere [1]. This environmental concern led to find sustainable alternatives for industrial processes, driven by the final consumer's awareness on this matter. This stakeholder is now open to the idea of paying more for a product that it is not so damaging for the environment. Therefore, it must be consider not only economical reasons but also ecological aspects in order to progressively replace non-renewable fossil stocks [2, 3] by changing the focus to the develop of alternative energy production platforms and producing chemicals using technologies capable of using biomass as a substrate [4]. Exploring a cheap, clean and renewable energy source has become a common goal since the 1970's fossil fuel crisis in which, as nowadays, the global economy was highly dependent on petroleum-based energy sources [5]. With the objective of shifting from fossil-based products, the hydrocarbons economy, to a bio-based raw material empowered economy there is some main topics that must always be taken into consideration when studying new sustainable technologies, namely: (1) the increasing energy demands; (2) accumulating atmospheric CO_2 from the burning of fossil feedstock; (3) energy security; (4) stability and sustainability and (5) the development and protection of the rural economy regarding the balance between the direct competition with animal and human food crops [5]. In response, many nations have funded extensive research and development programs so a sustainable and renewable solution could be achieved [5].

Initially, the 1st Generation Biofuels (1GBF) - which use food crops as raw-material, with traditional 1st Generation Sugar Production (1GSP) platforms - were presented as a solution. However, concerns about the feedstocks sustainability have risen, including the impact it may have on land use since the feedstock will compete directly with the food chain crops which originated the increase of the food prices in the past [5,6]. There is also concerns about biodiversity impacts in communities such as destruction of natural life by this unfair competition between crops used for biofuels production and the natural habitats. More specifically, it is also discussed that bioethanol and biobutanol for gasoline and diesel mixtures, respectively, are not an efficient CO_2 emission abatement technology. This means that to achieve the goals of carbon neutrality in the future, the fixation of carbon and subsequent reduction on greenhouse gases emissions, it is necessary to have a more sustainable technology [6].

Lignocellulosic Biomass (LCB) from agricultural and forest residues are an example of a sustainable, self-renewable and low-cost resource that can be converted into a large spectrum of products including fuels and chemicals on a large scale due to its high content of polysaccharides [1]. Approximately 200×10^9 tons of LCB are produced every year worldwide making this the most abundant renewable biological resource on earth [1, 7, 8]. Organic agricultural wastes (agricultural byproducts) are by definition:

(1) renewable; (2) available in abundance; (3) source of fibers, chemicals and other industrial products; and (4) far less costly than other feedstocks (crude oil, natural gas, corn kernels, and soy oil) based on the price-energy ratio [5, 7]. Consequently, there as been an increase in LCB processing research, focusing particularly on forest and agricultural residues. The challenge that comes with using this type of raw materials consists on overcome the inherently complex and heterogeneous composition as long as its recalcitrance to conversion reactions [1, 2, 4].

Lignocellulose is composed by cellulose, hemicellulose, lignin, extractives and ash. Both the cellulose and hemicellulose fractions are polymers of sugars and thereby a possible source of fermentable sugars. Lignin and the organic and inorganic components usually referred as extractives and ash, respectively, can be used for the production of chemicals, combined heat and power or other purposes [8].

With the usage of LCB raw-materials in the bioproduction technologies came the concept of 2nd Generation Biofuels (2GBF) where the direct conflict between this type of substrate and the food chain is avoided and so, by this, accomplishing the objective of global food security [9], but, the use of biomass as a resource for energy and fuel production is limited by maximum production rates and the supply of biomass, and so, nowadays, the technologies in place can not overcome the high energy and fuel demand [4]. With the actual available scientific know-how, even if all the worldwide LCB was only used to produce energy or fuels, it would only cover 20 % of the actual global demands [10]. The relatively low energy content, seasonality and non-uniform geographic availability of LCB have been identified as major obstacles to the large volume production of bioenergy and biosubstances [11], when compared to the traditional chemical production.

The overall goal of a bio-based production complex is the generation of a variety of goods from different biomass feedstocks through the combination of different multi-step hybrid technologies from different fields of research including polymer chemistry, bioengineering and agriculture. The end goal, or the ideal biorefinery concept, is to integrate, in the same biomass platform, conversion into fuels, power, biomaterials and biochemicals, allowing also the development of waste valorization procedures in order to get as much value of as possible from all the outlet streams [12]. In this concept, the term "waste" as something to discard or deposit is completely obsolete and it should be rethinked and looked at as a resource for further valorization [4]. Following this driving force, this is the current aim of the Bioactive PhD Program at TU Wien, more specifically, the development of a sustainable production process for pharmaceuticals as value-added products. This project, illustrated in fig. 1.1, relies on cooperation between all the investigation branches in order to achieve a sustainable and profitable process. This work main focus is in the pulping and extraction branch of the biorefinery, PhD 4 and 5, which consist in using some renewable feedstocks (in this work's case, wheat straw) to extract value-added products (bioactive compounds) and further using the remaining parts of the plant to produce nutrients for the bio-processes, energy and evaluating other possible products.

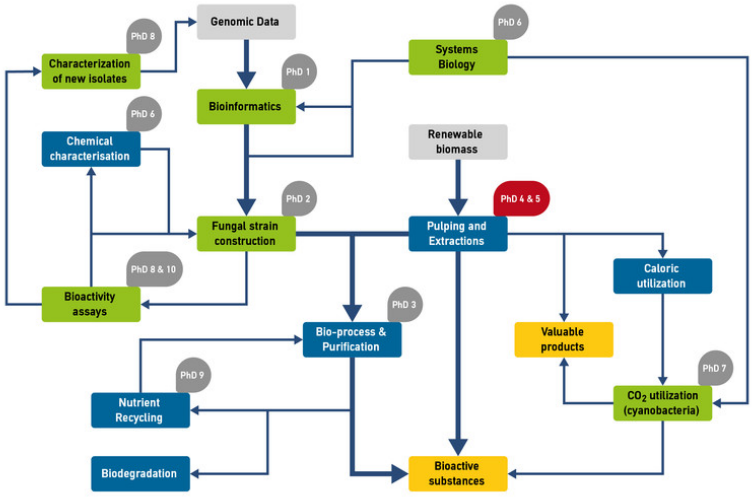

Figure 1.1: Bioactive PhD program representation in a blocks diagram. This work main focus is inserted in highlighted branch, the pulping and extraction, within the biorefinery concept.

The bioactive project uses, as substrate for the cultivation, mono- and oligomeric sugars that are extracted from LCB raw-materials. Additionally, the bioactive extractives from the plant biomass (fungal and herbal) will be screened for their bioactivity for further valorization and maximum utilization. Continuing to always have in mind the ecological footprint of the whole process and how to minimize it, it will also be utilized the plant biomass for the production of other valuable products (e.g. Nano-Lignin) and recycle inorganic nutrients for the cultivation. The final step will be take the remaining solid residues from the pulping process and the cultivation that can not be further valorized and fed them to a caloric unit, which will deliver the energy necessary for the pulping and cultivation processes. Concerning the metabolic gaseous stream, the carbon dioxide that is generated during the combustion and the cultivation will be bound by a cyanobacteria expression platform, which will be used to produce other valuable products and even more bioactive substances.

The purpose and aim of this master thesis work is to study the first step of biomass treatment right after raw material selection, which involves make it more amenable for sugar production, the pretreatment step, using a combination of a variety methods, being them physical, physicochemical, chemical or biological. Previous works have been done in order to characterize the so called 2nd Generation Sugar Production (2GSP) platforms in terms of processing, advantages and disadvantages of several combinations of protocols, sugar profile, etc., namely: Pentoses (C5)/Hexoses (C6) ratio, and inhibitors concentration, with aim to a future fermentation and its specific requirements [13].

In short, It is well justified to pursuit this economic opportunity for the development of bio-sourced chemical products since this market niche value is comparable to the fuel industry, but only requires a fraction of the biomass [4]. This work motivation is focused on acquiring if wheat straw, an agroindustrial

residue, can provide the sugar extraction concentration and profile for specific fermentation processes with a threshold of cell growth inhibitors by changing the operation conditions of pretreatment that this LCB will be submitted. If this goal is achieved, further developments will consist in conducting a cost-effective sugar production study, having in consideration nutrient recycling, waste stream management, and carbon dioxide valorization in order to minimize the ecological footprint of the process.

1.2 Bioactive project overview

This section is meant to clarify the objective of this thesis work in the Bioactive Project context.

Despite many bioactive substances are already being used in the pharmaceutical industry there are still many unknown biocompounds that can be used as such. The Bioactive program, as a hole, consists on enlisting how studies on these substances production, via fermentation, and further investigation in their specific substrate requirements can help build a sustainable and profitable process network.

This work main focus will be then assessing, in this context, if the raw-material of interest, wheat straw, is able to provide enough sugar concentration for further fermentation steps without having a inhibitory concentration of sugar degradation products within the supply mixture.

1.2.1 Scientific, technological and economical background

Microorganisms, such as fungi, produce bioactive substances via the secondary metabolism. The secondary metabolism is not mandatory to cell growth, being, as its name suggests, secondary to the primary metabolism, and only expressed under certain specific culture medium age. The secondary metabolism is unique in every fungus and consists of different metabolic pathways that result in a variety of chemical compounds, called metabolites. In fact, most of these pathways are not active under standard *in vitro* cultivation conditions, which hinders the discovery of new bioactive substances and the production of unknown compounds.

The discovery of this yet undescribed bioactive substances and its optimal production conditions is currently the biggest challenge of the pharmaceutical industry. During industrial productions, expensive and complex cultivation medium conditions need to be applied to induce synthesis of bioactive substances. Plus, once achieved, the fungi produce not only the desired compound, but also many other toxic and cellular growth inhibitory metabolites. Some fungi are even pathogenic, and their handling and cultivations follows restrict safety procedures, compliant with Current Good Manufacture Practices (cGMP). The current strategy for strain optimization is random mutagenesis and high-throughput screening. Needless to say, this method is highly laborious and time- and resource-consuming.

Additionally, current production methods tend to do not take ecological aspects into account. It is widely accepted in the scientific and industrial community that the medical end of the final product is

considered enough to justify all sustainability disadvantages of the current substrates that are used and the production process. Put in another words, any necessary substrate or production process, regardless of its ecological footprint or ecological impacts, are considered accepted, as long as the desired compound is produced in a cost-effective and profitable way. However, with many countries establishing regulatory frameworks and restrictive tax systems in order to promote sustainable production processes, this paradigm tends now to change. Further, the increasing number of expiring patents, making path to the rise of generic medical products, forces the pharmaceutical industry to develop more efficient and cheaper production processes, such as, employing concepts of reuse, recycling and use cheap feedstocks, as it is the LCB, the main objective of the present studies. By this manner, the study of LCB as source of nutrients for fermentations is well justified in the context here described.

1.3 Research goals

Focusing on the issue of environmental sustainability it is necessary to make a proper process mindset shift in terms of raw materials, meaning, go from the fossil-mineral oil to the renewable biomass of agro-industrial residues. LCB feedstocks are the most promising substrate to implement in large scale operations, since it is an inexpensive and abundant source of sugars that can be used for the production of numerous bio-based added value compounds such as biofuels and biochemicals [14]. With no direct competition with food crops it presents itself as an environmental and sustainable solution to be considered. It is this work objective to assess if the supply of the raw-material of interest, which is wheat straw, not only in terms of quantity worldwide but also regarding its availability in different world regions. LCB pretreatment still is one of the most expensive steps of the overall feedstock conversion to bioproducts and this lack of an efficient and low-cost technology to overcome biomass recalcitrance is an important matter to overcome that is preventing the more widespread use of LCB materials. Having this in consideration, this work will focus on evaluating the hemicellulosic sugar production efficiency of the Liquid Hot Water (LHW) pretreatment in order to be implemented into a biorefinery concept, and so it could become part of robust and feasible large scale operation [1]. The application of this pretreatment results in the formation of fractioning degradation products that inhibit microorganisms cellular growth. This compounds must be studied as well to assess the resulting sugar solution applicability in fermentations. Lignin is also solubilized by the LHW, or autohydrolysis, procedure. Being a value-added product, this convertion needs to be also studied in order to evaluate the LCB value that is being lost in the sugar solution fraction. Taking this criterium into consideration, this work will state which operational condition achives the sugar production requirments, maintaining the concentration of degradation products to a minimum, as well as lignin and cellulose within the solid fraction. This kind of research in the biorefinery area is considered to be the key for a successful integrated production of food, feed, chemicals,

materials, goods and fuels in the future in order to have achieve and environmental and economically sustainable process [15].

1.4 Organization of the document

This thesis is organized as follows: In chapter 1 an overview is given about the concept in which this work is inserted, as the interests and possibilities for developing a bioeconomy and how using LCB as feedstock is interesting to achieve the goal of a sustainable cost-effective process. In chapter 2 it is presented a concise bibliographic review, describing not only the technological and scientific background of the biomass refinery concept but also focus on agricultural residues, their composition and biotransformation route within the processes including pretreatments, conversion and final bioproducts profile. It is presented also, some of the different types of previous works and the hypoteses for sugar production implementation, using biomass, and examples of various pretreatments for the development of a bio-based economy all over the world taking into account local adaptations for each region´s available feedstock. In chapter 3 it is well described the experimental protocol of the LHW process that took place in this work, as well as any prior and posterior procedures, for sample preparation and analysis, respectively. This protocol was constructed in order to achieve reliable, reproducible and concise scalable results for future implementation in the biorefinery concept. Chapter 4 presents those results it in order to assess if all the hypothesis presented in the previous chapters are valid or not. If the results need a deeper assessment or lead to other hypotheses this is presented in the chapter conclusions and further perspectives, being chapter 5.

2

State of the Art

Contents

2.1 Biorefineries

A biorefinery is a complex system that aims to respond to the nowadays world demands in terms of bioproducts for chemical industry.

Due to the concerns presented previously in chapter 1, the biochemical industry has been discovering the applications value of lignocellulosic residues into biorefineries, namely, to their abundance, prince and availability worldwide [16]. A promising area in terms of sustainability has been then developed and it is generally refereed as the LCB feedstock biorefineries. [17]. A biorefinery is usually referring to use of renewable materials and their residues, in a most integral and diversified way for the production of fuels, chemicals and energy, with minimal generation of waste streams and pollutant emissions [16].

The most exhaustive definition was made by the International Energy Agency (IEA) and states: "Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy" [17].

The biorefinery concept is analogous to the traditional refineries which produce multiple fuels and products from petroleum and where an industrial segment works as a generating pole of raw materials to others [18]. By changing the input raw material - from petroleum to biomass - and making use of various hybrid operations, this network of biomass substrates, intermediate substances/energy and main products, ideally, form a framework of waste-free streams with total valorization of each unitary operation outlet current [16] (see fig. 2.1).

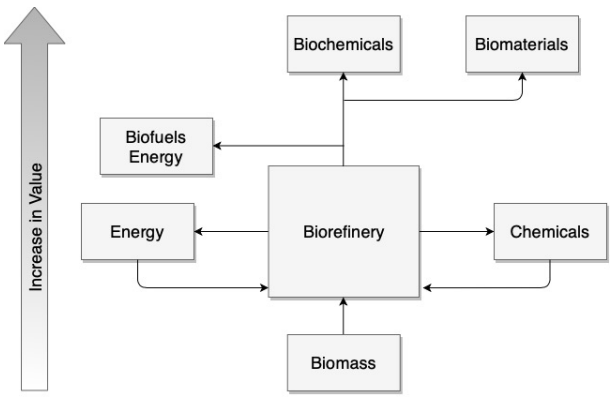

Figure 2.1: Biorefinery concept, adapted from Carvalheiro et al. (2008)

2.2 Lignocellulosic biomass

LCB is an example of a sustainable, renewable and low-cost resource that can be converted into a large spectrum of products including fuels and chemicals on a large scale due to its high content of polysaccharides [1]. Lignocellulosic plant biomass consist mainly cellulose, hemicelluloses, and lignin,

as well as extractives and ash content, although in smaller amounts.

It is well known that the content fraction of this various components depends on the type of biomass, the plant age and also the source´s region [1]. For instance, forest products contain higher cellulose content than agricultural residues, but, on the other hand, these lasts contain a higher level of hemicellulose and a lower amount of lignin than wood materials [19]. Furthermore, low age wood contain mostly lesser amounts of all structural components (cellulose, hemicellulose and lignin) than older woody materials [20].

The LCB agricultural by-products that are available in considerable quantity and at low cost are, among others, corn stover, wheat straw, maize, rice, barley straw, sorghum stalks, coconut husks (coir), sugarcane bagasse, and pineapple and banana leaves [1, 7]. Using these crop residues for industrial applications could be an additional source of revenue for farmers, without adversely affecting soil fertility or competing directly with food crops. For example, LCB can be applied for cellulosic material recovery or sugar production, such as paper and cardboard, both having a high content of cellulose [14].

This renewable plant waste is abundant, biodegradable, low cost and low density that could be a principal source for production of fibers, chemicals and other industrial products. The uses of these materials are not only limited to composite, pulp and paper industry or textile applications, but are also progressing immensely to many other unlimited applications such as medical, nanotechnology, biofuel and pharmaceutical. [21]

Notwithstanding, the recalcitrant nature of these materials - which means the ability of the plant cell wall to resist deconstruction - makes their utilization a challenge [1] and will be further discussed along this work specially in section 2.3.

2.2.1 Diversity and availability

With the substrate change from food crops to LCB, it is necessary to take into account local biomass supplies in terms of availability and diversity so a sustainable process can be constructed. Some examples of LCB to be considered can often be described as: (i) forest residues - which include broadleaf woods from the most various species of higher plants, softwood (pine, redwood and spruce) or hardwood (eucaliptus, oak and walnut) and also grasses. After processing, the remainings of each stated LCB source is also considered to be part of this category [22]; (ii) agricultural residues - for example late grass, reed, bush and the harvest remains such as wheat straw, corn cobs, barks, stems and tree pruning [14]; (iii) Municipal Solid Waste; (iv) agro-industrial by-products including sugarcane bagasse [23], brewery's spent grain [24, 25] and also black liquor, a resultant residue from the pulp production process [21]. It is worth to remark that the use of crop residues is especially interesting because it helps also with the problem of this type of waste management.

2.2.1.1 Lignocellulosic biomass sources

Studying several sources of LCB, such as local crops and forest, is crucial to observe which type of raw-material exists in a certain region and when it will be available for exploration.

Wheat was the 10th most produced crop worldwide in 2018, with a total production of around 734 million tons, making 25% of the total cereals production.

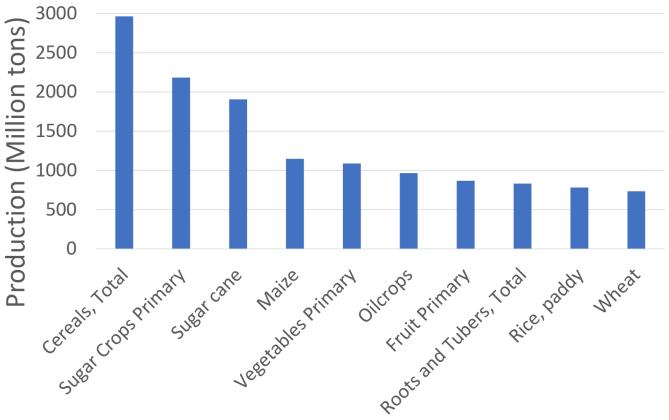

Figure 2.2: Ten most produced crops worldwide. Adapted from FAOSTAT (2018)

Lignocellulosic crop residues are interesting raw-materials for a biorefinery concept, being relatively cheap and available in the nature. The most abundant crop residues include straw and stubble from cereal crops, such as wheat, barley, sorghum and oats. There are also available stalks and leaves from corn, usually know as corn stover. It is important to emphasize that this crop residues must be partially lefted on the crops field thus avoiding soil dryness, retaining of water and organic matter, maintaining biological activity, and minimizing erosion by the wind and rain effects. All this factors will allow the agricultural field to remain viable for further crops plantation and cultivation.

The next table, table 2.1, shows how the general ratio between the amount of residue possible to obtain from the original crop, giving an primarily estimation of the available LCB from that specific source.

Table 2.1: Residue to product ratio (mass residue/mass of product in dry basis). Specifically for wheat straw, this ratio is often mentioned as residue-to-grain. Adapted from Fialho (2015).

Product	Residue to product ratio
Wheat	1.5
Oat	2.0
Corn	1.0

Using the ratio stated by Fialho (2015) [26], for wheat, there is around 1101 million tons of wheat

residues which is 37% of the total cereal production around the world. So it is more that justified to study the use of this residue as substrate on biotechnology processes since it is highly available around the world.

2.2.1.2 Wheat crops production distribution

Specifically for wheat straw, it is of interest to assess this crop annual production, land use and its role in the cereal industry, not only worldwide (fig. 2.3), but for the countries of Austria (fig. 2.4) and Portugal (fig. 2.5).

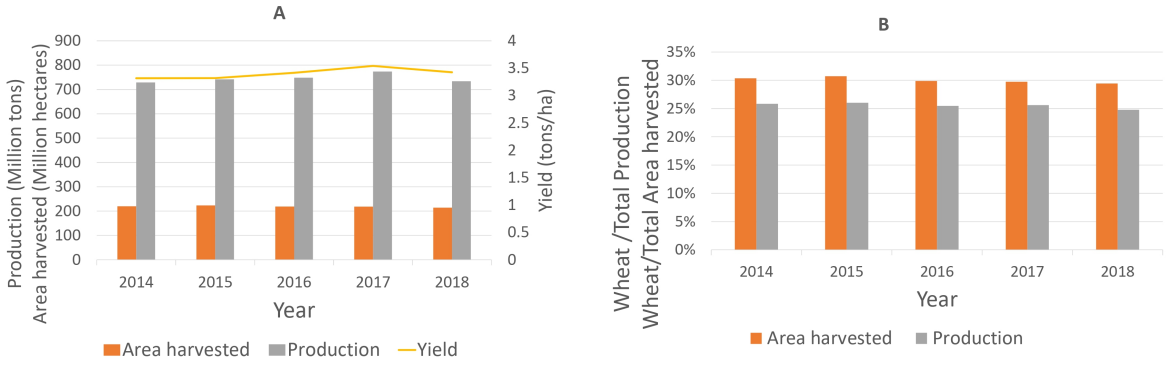

Figure 2.3: **A**: Evaluation of the annual production (left axis), area (left axis), and yield (right axis) for wheat straw worldwide. **B**: Evaluation of the wheat straw annual production contribution to the total cereal production worldwide and wheat area harvested contribuiton to the total cereal area harvested worldwide (both in percentage). Adapted from FAOSTAT (2018)

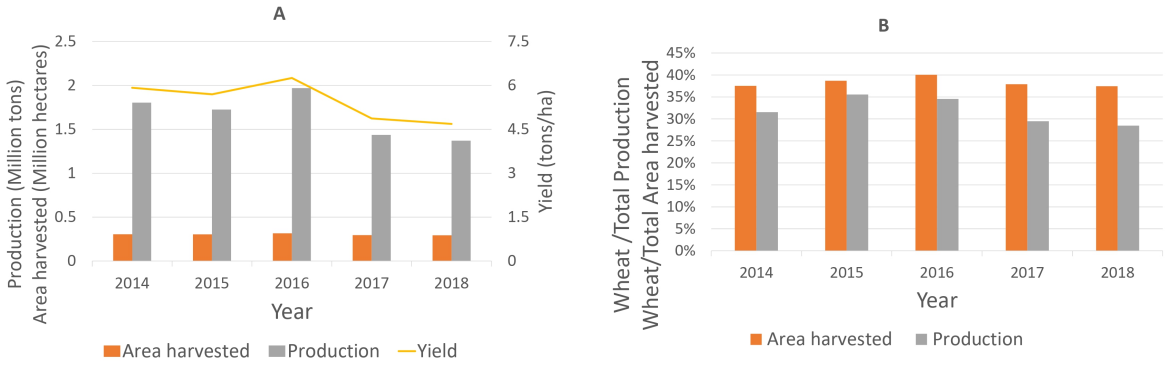

Figure 2.4: **A**: Evaluation of the annual production (left axis), area (left axis), and yield (right axis) for wheat straw in Austria. **B**: Evaluation of the wheat straw annual production contribution to the total cereal production in Austria and wheat area harvested contribution to the total cereal area harvested in Austria (both in percentage). Adapted from FAOSTAT (2018)

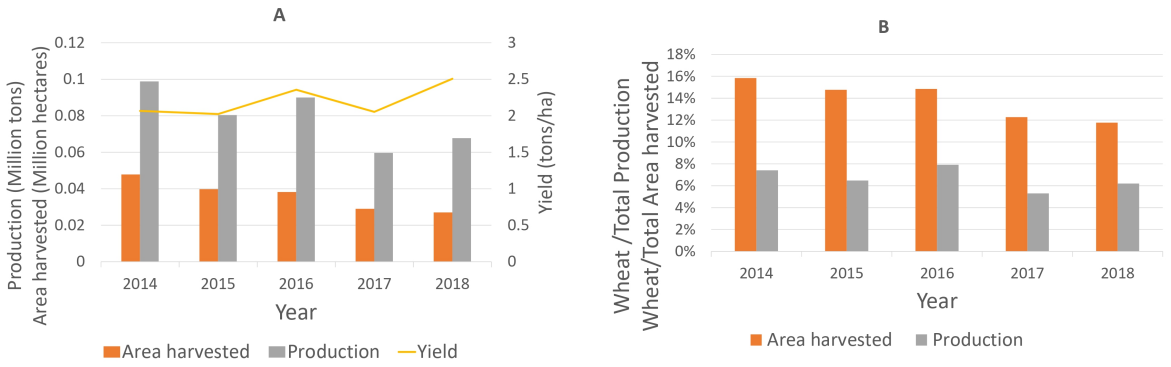

Figure 2.5: **A**: Evaluation of the annual production (left axis), area (left axis), and yield (right axis) for wheat straw in Portugal. **B**: Evaluation of the wheat straw annual production contribution to the total cereal production in Portugal and wheat area harvested contribution to the total cereal area harvested in Portugal (both in percentage). Adapted from FAOSTAT (2018)

The worldwide production parameters were maintained stable during from the year 2014 to 2018, which means that this crop might present a fixed value and high presumable availability, not only regarding the food crop but also the consequent wheat residue.

In Austria, despite the decrease in harvest yield and wheat production, it can be considered that wheat straw sill contributes to the agro-industrial production of cereals since it is next to 30% of the total cereal, in 2018, production in the country, well above the worldwide contribution (25%) which means a local production of 1.4 and 2.1 million tons of wheat and residue, respectively, guaranteeing its production and local LCB supply.

For Portugal, there as some challenges regarding local supply of wheat straw residue. The Portuguese wheat crops represent only around 6% of the total cereal production, far away from the worldwide value, corresponding to the annual production of around 0.07 million tonnes (67749 tons) which corresponds to a 0.1 million tons of residue. The Portuguese wheat straw is around 5% of the total Austrian production, in 2018, despite the yield increase for this year. If an oscillation from 8% to 6%, as it was from 2016 to 2017, happens, since the wheat straw contribution is too low, this translates in a decrease of around half a million ton of wheat straw available to be used.

Since the biomass refinery project is meant to be implemented in Austria, the concerns about LCB supply can be considered to be well handled, however, if a transposition to the Portuguese context is to be made, it might be necessary to consider another local raw-material, that gives a more predicted price and supply.

2.2.2 Composition and structure

It is impossible to assess the capacity and limitations of the different biofractioning technologies without knowing first the LCB composition and structure. As stated in section 2.2, plant biomass consists of in cellulose, hemicelluloses, lignin, extractives and ash content. In this subsection it is meant to explain and illustrate the composition and structure of LCB.

2.2.2.1 Cellulose

Cellulose is a major structural component of the cell walls providing mechanical and chemical resistance to plants. It is formed as a stored product of the photosynthesis process due to solar energy absorption [27]. The chemical formula of cellulose is $(C_6H_{10}O_5)_n$, in which n is the degree of polymerization, and the name for it is the 4-O-β-D-glucopyranosyl-D-glucose, also known as β-1,4-polyacetal of cellobiose. Cellulose is a polymer of glucose because cellobiose consists of two consecutive glucose monomer molecules [8]. The bond nature between the glucose molecules (β-1,4 glycosidic) allows the polymer to be arranged in long, stable, homogeneous, long straight chains.

Cellulose is found in both crystalline and the non-crystalline (amorphous) arrangements due to the coalescence of several polymer chains caused by the fact that hydroxides are evenly distributed on both sides of the monomers which allows the formation of hydrogen bonds between cellulose chain molecules [28]. Crystalline regions, which are harder to be hydrolyzed than amorphous regions hinder the enzymatic or chemical monomerization of cellulose and render it insoluble in most solvents [1]. This hydrogen bonds give rise to the formation of several parallel chains attached to each other resulting in the formation of a crystalline structure called *microfibrils*, which, in turn, gather together to constitute *fibrils* and subsequently are united to form cellulose fibers [1, 8].

In terms of the cellulose physical and chemical characterization, being an organic polymer tends to be an hygroscopic material and insoluble in water, where it swells, absorbing between 8 and 14 % water under Normal Temperature and Pressure (NTP) conditions (20 ℃, 60% relative humidity) [8, 29]. For instance, it is plausible to think that the cellulose polymer chain, with temperature increase, happens to be enough energy to break former intracellulosic hydrogen bonds and form new ones with water molecules, becoming soluble in an aqueous environment [8, 30]. It is worth to remark that cellulose does not melt at NTP conditions, however it starts to decompose into a variety of cellular growth inhibitors at approximately 100 ℃ [31, 32].

2.2.2.2 Hemicellulose

Hemicellulose represents a family of polysaccharides that are found in the plant cell wall and have a different composition and structure depending on their source and the extraction method [8]. It is

also the least stereochemically impeded polymer being even more exposed to react if lignin is primarily removed [1].

The most common type of polymers that are part of the hemicellulose´s polysaccharides is xylan but there is other examples like arabino-xylans, gluco-mannans and galactans [8]. The xylan molecule involves β-1 $\rightarrow$ 4 linkages of xylopyranosyl units with α-(4-O)-methyl-D-glucuronopyranosyl units attached to the anhydroxylose main chain units resulting in a polymer that is composed largely of C5 sugar monomers, D-xylose (about 90%) and L-arabinose (nearly 10%) [1], and to a lesser extent C6 sugar monomers such as glucose, mannose and galactose [8]. Hemicellulose bind to cellulose *microfibrils* by hydrogen bonds and to lignin via covalent linkages. Unlike cellulose, hemicellulose present a variable and amorphous structures of short side chains along the hemicellulose main backbone chain that can be easily degraded by enzymatic hydrolysis and/or chemical treatments. This complex characteristic aspects of the hemicellulose´s structure and composition are important to understand the lack of a crystalline matrix specially due to the highly branched molecule organization and the presence of acetyl groups connected to the polymer chain [33].

Hemicellulose, as cellulose, is insoluble in water at NTP conditions, and its hydrolysis starts at 100 ℃ as well [31]. Also, the presence of acid highly improves the solubility in water, as it is explained further in section 2.3.1. Hemicellulose, when pretreated, has shown a tendency for sugar degradation mainly derived from the pentoses present in their constitution [1]. Hemicellulose also contains a high degree of polydispersity, polydiversity and polymolecularity which means a broad range of size, shape and mass characteristics [8].

2.2.2.3 Lignin

Lignin is the most complex natural polymer and is the main responsible for the LCB recalcitrance. Due to its reticulation and hydrophobicity, lignin is insoluble in water or aqueous solutions, giving it the ability of liquid transportation and waterproofing. The irregular and heterogeneous lignin structure provides rigidity and physical strength to the biomass cell wall creating mechanical endurance, resistance to impact, compression, bending, degrading chemical agents and microbial attacks by binding the material between cells [26, 34]. Lignin is insoluble in most solvents with the exception of low molecular alcohols, dioxane, acetone, pyridine and dimethyl sulfoxide [8]. Lignin can also be submitted to elevated temperatures in order to suffer thermal softening prior to acidic or alkaline reactions in order to improve kinetics [8]. It is an amorphous three-dimensional polymer with phenylpropanoid units as building blocks [1, 8]. As mentioned before in the beginning of section 2.2.1, dividing higher plants into hardwood (angiosperm) and softwood (gymnosperm), it has been identified that lignin from softwood contains more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol units. On the contrary, hardwood lignin tho is made up of several different ratios of coniferyl and sinapyl alcohol [27]. This units

are interconnected by different types of bonds including β-O-4-aryl ether and resistant C-C bonds. The corresponding monomers of the phenylpropanoid units have been identified as guaiacyl, syringyl and p-hydroxyphenyl units [8, 35]. Lignin connects with hemicellulose and cellulose through covalent bonds via ester, ether and glycosidic linkages and the lignin molecules also enclose these polysaccharides, hampering their access [1].

The lignin composition presents variability with the source of the LCB. For example, grass lignin is made up of all three monomeres (guaiacyl, syringyl and p-hydroxyphenyl). On the other hand, lignin from softwood is mainly composed of guaiacyl units while lignin in hardwood contains a high amount of syringyl units. This ones determine the structure and characteristics of the hardwood lignin since the presence of the methoxy groups in positions 3 and 5 of the aromatic ring do not allow the formation of ether and C-C linkages between the monomers units which ends on the reduction in the polymer chain length and condensation. For this, hardwood are easily delignified than softwood [36].

2.2.2.4 Extractives

Although LCB is manly composed by the previous components described before in this section, a minor fraction of organic components is found within biomass. Unlike the ones mentioned before, these substances do not provide a structural function to the biomass but act as metabolic intermediates, energy reserves or as a part of the defense mechanisms against microbial attacks [1]. These components are often referred as extractives and constitute a heterogeneous group that includes, among others waxes, fatty acids, gums, resins, chlorophyll, terpenoids and a variety of phenolic substances. Examples of organic solvents that are able to solve this substances are (1) ethanol; (2) acetone; (3) dichloromethane; and (4) benzene [1]. This components are the main responsible for LCB characterization in terms of color, smell and resistance to wilt [37].

2.2.2.5 Ash

LCB also comprises minerals present in structural or extractable components. These minerals include, among others, Ca, K, Mg and Si [38]. On average, softwood and hardwood contain about 0.4 %wt and 0.5 % wtof dry matter respectively, while the ash content of fibrous agricultural crops ranges from 1%wt to as high as 20 %wt of dry matter [39]. Serna et al. (2020) [40] stated an ash content for wheat straw of 1.09 %wt.

2.2.3 Sustainable production of lignocellulosic sugars

In order to achieve the goal of process optimization, it will be studied and compared different extraction methods and different biomass sources to maximize the nutrition yield, while minimizing the energy

and chemical demands of the extraction process.

Understanding of the chemical composition and structure of natural lignocellulosic materials, characteristics of each component, and interrelationships between various components would contribute to the research and development even greener technologies regarding natural LCB.

Therefore, the main objective is to extract sugars from LCB as well as other nutrients needed for cultivations with the goal of achieving optimal nutrient concentrations for cellular growth. Hemicelluloses are stereochemically most accessible so the main focus in this thesis experimental work will be to analyse not only the C5 sugars concentration originated from this polymer but also their relationship with other C6 sugars originated from both hemicellulose and cellulose and try to find the optimal relationship between obtained sugar profiles and the extraction method conditions that will be used. The type of products, namely sugars, extractives and polymers are highly dependent on process conditions and specifically which kind of pretreatment is the substrate exposed to.

In previous works, cellulose has been treated as a source of monomeric glucose for further fermentations, despite of being, by itself, a high-value lignocellulosic product. The process valorization technique often involved transforming this sugars into bioethanol. However, bioethanol from LCB has a way cheaper market competitor - bioethanol from brazilian sugar cane. Since the labour and raw-material costs in this process are far smaller than in the european context, the need of a change in the focus of sugar extraction imposes by itself. One of the solutions explored in this project is maintaining, as far as possible, the cellulose structure, ergo, only removing hemicellulosic sugars. If this the extracted sugars profile and concentration are enough to fulfil the substrate needs for a microorganism of interest a product valorization is achieved in both fronts, which is the final goal of a biorefinery. Following this path of further valorization of LCB raw-materials into high-value products by using polyssaccharides into fermentable sugars, LCB can also be used in more attractive and profitable way, which means, production of solvents, food protein mass and other added-value metabolites such as vitamins (aspartic acid or glutamic acid), sugar alcohols (polyols, such as glycerol, xylitol, arabitol, erytritol and sorbitol) or organic acids are also an option [1].

In a symbiotic waste-free perspective, the aim is to use as much of the remaining components of the plant biomass, which means, cellulose and lignin, proteins and amino acids, fats and fatty acids, resins, tannin, and many different aromatic compounds, to yield valuable products by extraction.

After the cultivation and product purification, two streams of residuals will arise, the solid fungal biomass and liquid residues. As stated before, the aim is to recycle inorganic nutrients and salts from these streams to be reused for the cultivation. Unusable fungal biomass will be subjected to a caloric utilization, together with unusable remains from the plant biomass extraction processes. The energy that is released will be used to cover the energy demands of the pulping and cultivation processes. This process network also contemplate a gaseous stream valorization with the objective of fixating the

metabolic released carbon dioxide in a cyanobacteria platform, because they possess a huge application possibilities for a vast number of microbial processes and products.

In order to close the sustainability cycle, the remaining liquid stream will be subjected to biodegradability tests. The resultant bioactive substances, of the posterior integration into the biorefinery concept, with intended mode of actions are the goal for use in medicine, the same rigorous approach may be considered as undesired effect and pollution in the environment receiving wastes. A relevant selection of tests will be applied to evaluate the treatment technologies considered for final waste treatment so as to minimize or even annul the environmental impact.

2.3 Pretreatment of lignocellulosic biomass

LCB raw material pretreatment is basically opening up the structure of a plant cell wall so that catalytic reactions can occur. Since there is a natural resistance to deconstruction in this materials those need to be exposed to conditions out of the normal and natural range of the atmospheric environment allowing lignocellulosic biomass to be vulnerable to the attack by the catalytic molecules. It is an essential first step in the overall conversion of biomass into bio-based products and biofuels [1, 2, 41, 42].

In order to accomplish an effective and efficient utilization of lignocellulosic raw materials and so achieve a robust, independent and reliable process for a biorefinery, the biomass fractionation in its main constituents must be extensive and as complete as possible. That is only possible by overcoming the recalcitrant nature of the LCB as mentioned in the beginning of section 2.2. This procedure of submitting the feedstock to a pretreatment stage is considered the most important and limiting step for obtaining an efficient conversion of biomass into highly digestible solids that enhance sugar production. The pretreatment allows the disruption of the cell wall physical-chemical barriers in order to facilitate hydrolysis reactions of cellulose, hemicellulose and lignin, by (a) solubilizing lignin and hemicellulose fractions; (b) reducing cellulose crystallinity; (c) swelling pores in the biomass structure; (d) and increasing the available surface area [1]. As a result, the sugar matrix is able to be more easily accessable substrate for sugar solubilization. Factors such as the cellulose crystallinity, the protection exerted by hemicellulose and lignin fractions and the accessible surface area of cellulose hinder its digestibility [43, 44]. The effect of pretreatment is illustrated in fig. 2.6.

The use of pretreatment steps, despite allowing sugar solubilization, has the major disadvantage of producing fermentation inhibitory components, usually called, pretreatment degradation products. This degradation products are mainly acetic acid, 5-hydroxymethylfurfural (HMF) and furfural. The first component has its origin by the detachment of acetyl groups from the hemicellulosic matrix, even at temperatures bellow the ones that hemicellulose starts to be hydrolysed. The HMF and furfural are decay products from C6 and C5 monomeric sugars, respectively. This components production is highly

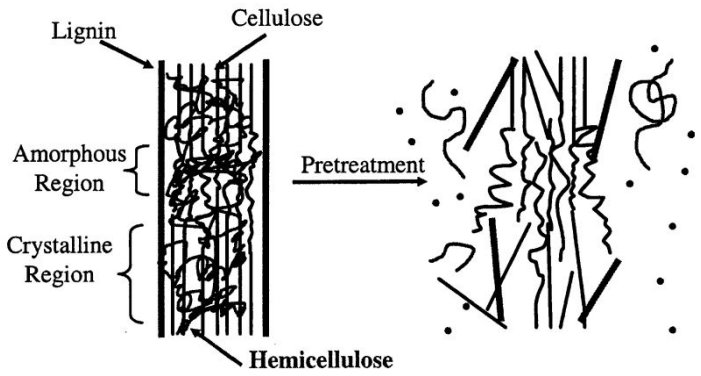

Figure 2.6: Effect of pretreatment in the lignocellulosic matrix, adapted from Mosier et al. (2005)

affected by the pH, since the formation of acetic acid catalyses the hydrolyzation, not only of the carbohydrates matrix, but also the correspondent monomeric sugars. [1, 32]

The pretreatment is considered in the most expensive steps in a biorefinary so, in order to be attractive in a cost-effective point of view it must be done in moderate size bioreactors and minimizing heat, power and chemical requirements [1]. Pretreatment is one of the most expensive steps within the overall process of lignocellulose conversion to bio-based products. For example, in ethanol production, economic analyses indicate that the greatest cost parcel is associated with the stages related to the production of fermentable sugars from cellulose and hemicellulose (almost 40 %) involving pretreatment steps, enzyme production and enzymatic hydrolysis, being the pretreatment almost 18 % of the total production costs - half of the sugar release stages cost [45].

As cited in the beginning of chapter 1, studies have been made in order to achieve effective and low-cost pretreatment methods to overcome the cost barriers for biomass utilization. To be effective, either technologically and economically, it should meet the following requirements: (1) deconstructing the three dimensional structure of LCB by reducing the polymer chain length; (2) increasing the surface area and porosity of the material; (3) generating highly digestible pretreated solids and promoting high sugar yields after hydrolysis (greater than 90%); (4) avoiding the formation of fermentation inhibitor compounds (specifically (a) acetic acid from hemicellulose (b) furfural and hydroxymethylfurfural from sugar degradation and (c) phenolic compounds from lignin degradation) [32]; (5) allowing hemicellulose and lignin recovery from subsequent use on production of valuable coproducts; (6) requiring a low demand of post-pretreatment operations and consumables such as washing and neutralization so it requires minimal energy, chemicals and water inputs using simple, reasonably sized low-cost bioreactors [1].

Biomass pretreatment processing can be divided into the following categories: (a) physical, which aims to increase biomass surface area and reduce the particle size and cellulose crystallinity; (b) chemical, that typically focus on lignin removal and the swelling of crystalline cellulose; (c) physico-chemical,

and (d) biological, which is applied in order to degrade long polymer chains into fermentable sugars with high yields and low inhibitor production.

2.3.1 Acid Hydrolysis

Treating LCB with acid solutions is one of the most employed methods for this materials pretreatment being a cheap and effective method of fractioning. The diluted or concentrated acid acts as a catalyst in the break of the LCB rigid structure, attacking intermolecular and intramolecular bonds in the material structure and hydrolyzing, mainly, the hemicellulose [46]. The hydrolysis of the hemicellulose can be achieved almost without damaging the cellulose polymer since the bonds in hemicelluloses are weaker than in cellulose due to its shorter structure, the presence of side chains, and type of linkages [47], and so, low degradation into cellulosic glucose is obtained when diluted acids are used even with elevated temperatures ($\approx$ 200℃) and pressures until 10 atm [46, 48, 49].

Several aspects are crucial to achieve proper LCB materials fractioning, such as, type of acid, solid loading, temperature, reaction time and acid concentration. Acid pretreatment can be preformed using either low ($<$5% w/v) or high acid concentration ($>$30% w/v). In this procedures, an inverse relationship between temperature and acid concentration is applied, since when diluted acids are used the set temperatures tend to be higher (between 120 ℃ and 210 ℃) [32, 49] than when this method is preformed with concentrated acids (temperatures usually set to be less than 100 ℃) [50]. Since hemicellulose, by having weaker bonds than the other components, is the first constituent of LCB structure to break down during acid pretreatment, being readily hydrolyzed in moderate conditions, such as diluted acids, moderate temperature (100-120 ℃) and atmospheric pressure.

In the case that the objective is to produce the most of fermentable sugars possible, in the biorefinery concept, both hemicellulose and cellulose sugar yields increased if concentrated acids are used under the conditions described above [50].

2.3.2 Liquid Hot Water

Liquid Hot Water (LHW), also know as autohydrolysis, is an hydrothermal treatments are based on water and/or steam usage to heat up the LCB raw-material of interest in order to solubilize hemicellulose and at the same time maintaining, as much as possible, the cellulose fiber matrix, for further valorization. Following the biorefinery concept described in section 2.1, the hemicellulosic sugars are also recovered and used, for example, as a substrate for cell growing of microorganisms that use C5 sugars in their metabolism. The main difference between these types of procedures and acid hydrolysis (see section 2.3.1) is that the sugars from hemicellulose are mainly recovered in oligomeric form, whereas in the acidic processes, the sugar´s polymeric chain suffer a more extended hydrolisation and so monomeric

sugars are mostly obtained as a result of this procedure [51], In this work, it is approached the LHW technique, in order to efficiently fractionate LCB using a cost-effective and environmental friendly procedure [52]. It is worth to mention that hydrothermal-based processes can and should be combined with other type of pretreatment techniques in order to achieve the ultimate end of an integral valorization of all LCB components [51]. The LHW pretreatment consists of submitting a mixture of LCB and water at a specific solid concentration - Liquid-to-solid Ratio (LSR) - to heat radiation at pressures above saturation point for given period of time.

The increase in the system pressure allows the mixture to rise the temperature without reaching the boiling point and so, ideally, without any losses due to evaporation. The operational temperature usually range between 150 [51] and 240 ℃ and pressures between 10 and 35 atm [52]. The operation time may depend on the temperature and the type of experiment being conducted, which means: (1) Isothermal or (2) Non-isothermal . The procedure (1) is characterized by having a holding time at a given temperature besides the heating and cooling times that both procedures need to have. On the other hand, in procedure (2) the heating is only conducted until the mixture reaches the desirable temperature. When that happens, the resulted product is cooled down as fast as possible. Differences in both procedures have been studied previously and compared having in consideration the Severity Factor (R_0), described in section 3.3.8 [53]. This parameter is calculated integrating the variables reaction time and temperature in one, so a quantification of the reaction harshness towards the raw material by both operational conditions can be made.

This pretreatment concept was firstly introduced to explain why the acids derived from the hydrolysis of the hemicellulose acetyl groups, as described in section 2.2.2.2, catalyzed the hydrolysis of hemicellulose-lignin bonds as well as the hydrolysis of polysaccharides. During the reaction of lignocellulosic biomass with hydronium ions generated from water ionization lead to hemicellulose depolymerization by both hydrolysis of the ether bonds and clevage of acetyl groups. The hydronium ions are then regenerated by the acetyl groups and take a catalytic role on this process. Therefore, autohydrolysis methods are more effective for materials that have a significant content of acetyl groups which is the case, for example, of hardwoods and agricultural residues.

2.3.3 Organosolv

Organosolv of lignocellulosic biomaterials consists in using organic solvents, usually mixed with water, for high-quality lignin extraction. The lignin extracted via organosolv steps is relatively pure, has low sulfuric content, and is less condensed than that produced by other lignocellulose pretreatments [54]. Some examples on the reagents used to perform this type of extraction are ethanol, methanol, glycerol, ethylene glycol, triethylene glycol, acetone, dioxane, and phenol [42]. The standard methodology is similar to the one used in LHW procedures, which means that it is also applied the concept of submitting the

reaction mixture (raw-material + diluted organic solvent) to certain level of severe conditions towards the biomass, conditions that are quantified by the severity factor, R_0, within the temperature range between 100 and 250 ℃ [26]. In the case of holding times, the intervals change with the type of experiment preformed, going from a null residence, or holding, time to hours [8, 55]. Usually, it is added a catalyst to promote the rupture in the lignin-hemicellulose complex, aldo, for temperatures above 185 ℃, it is believed that there is enough concentration already of acidic components, such as acetic acid, for the reaction to be autocatalyzed. In previous published works, Ruiz et al. (2011) [56] used for this, 0.1% sodium hydroxide while Mesa et al. (2016) [57] used instead 1% sulfuric acid in their extractions. On the other hand, no catalytic component was added to the reaction mixture in the cases of Weinwurm et.al (2012) [58] or Win et al. (2016) [59]. With this process application, lignin and hemicellulose are solubilized into the liquid stream while the cellulose remain in the solid fraction. This cellulose-enriched solid product is now ready to be used either for further degradation into glucose (by the application of other pretreatments, for example, enzymatic treatemnt, described in section 2.3.4, and consequent use in fermentations upstream, within the biomass refinery process flow, or to be taken as a value-added product itself, for example, for the pulp and paper industries [60]. The enriched hemicellulose and lignin liquid fraction can also be subjected to separation procedures in order to extract the value added products from within, such as high content of hemicellulosic sugars, furfural, HMF, and other extractives, but the main objective is to retrieve the high quality lignin.

It is stated by Zhao et al. (2009) [61] and Mesa et al. (2016) [57] that the use of this type of lignocellulosic materials deconstruction steps has the critical setback of requiring the provision of substantial quantities of expensive used solvent for the process. The higher operating cost and associated explosion and fire danger are the major reasons that have held back the industry scale-up for this pretreatments, which makes it unviable, at the moment, for large volumes. One solution that is being exploited and studied is the integrated recycling of the organic solutions used in the process. This is seen as a triple advantageous technology since allows the reduction in the inlet supply of fresh organic reagents, which means less operational costs, also protects the environment by reducing the outlet toxic compounds concentration on the waste stream, and it decreases the concentration of cellular growth, fermentation or enzymatic hydrolysis inhibitors in the liquid stream so it can be used for the already stated upstream valorization steps [61].

2.3.4 Enzymatic Hydrolysis

The process of the conversion of biomass-derived biopolymers, cellulose and hemicellulose, into fermentable sugars is known as hydrolysis. When specific cellulases and hemicellulases are used for this purpose, then it is applied an enzymatic hydrolysis step. This procedure has become of interest in order to replace impractical harsh chemical and mechanical treatments and its disadvantages in terms

of capital costs and environmental concerns. The main setback is to preform a profitable and, therefore, cost-effective protocol involving enzymes, since they are an expensive bio-catalyst [62].

Before starting the enzymatic procedure it is normally and widely done an *a priori* treatment on the LCB materials by the methods already here described (*vide* section 2.3.2 and section 2.3.1) or others presented in the literature [1, 60]. This is done, not only because of the natural recalcitrance of LCB, here described in previous sections, but also because of the high content of the other main components of LCB mateirals - hemicellulose and lignin - that highly influence the efficiency of the *a posteriori* cellulose hydrolysis. Also, it is known that oligosaccharides released by the hemicellulases have inhibitory effects on cellulase [63]. Lignin has been demonstrated to be the most limiting factor for the enzymatic hydrolysis of cellulose namely due to: (a) being a physical barrier, which reduces the accessibility of the cellulase to its respective substrate and (b) cellulase is non-specifically absorbed to lignin, which leads to a decrease in the exposure of the enzyme's active site to the substrate [64]. After the increasing of porosity by any fractioning methods LCB is then available and in reach of the biocatalysts active site, in order to, successfully, hydrolyse the LCB substrates with high fermentable sugars yields [8, 63].

Enzymes, in general, aim to have the following desirable characteristics: (1) catalytic efficiency, (2) thermal stability, (3) end-product inhibition resistance, (4) shear inactivation [63]. The catalytic efficiency, for instance, is highly dependent on the Degree of Polymerization (DP), since a high cellulosic crystallinity leads to a lower efficiency in the catalytic reaction owing to the inherent features of cellulose (see section 2.2.2.1). Thermal stability is also a characteristic to have in mind since thermostable enzymes can be used at temperatures higher than 37 ℃ [63] (and up to 100 ℃ [65]) and so facilitate the break down of the LCB structure, leading to an also higher rate of cellulose hydrolysis. It is worth to remark also that most cellulases are inhibited by the products (e.g.: glucose and cellobiose). If end-product resistant cellulases are used, it can be produced a more concentrated mixture of sugars, which will avoid the need of a concentration step in the Downstream Processing (DSP) before bioconversion in the added-value products of interest. Considering that basically ever pretreatment step in this fractioning phase of LCB treatment is done in a Stirred Tank Reactor (STR), shear-resistance cellulases will be useful where agitation is a must to provide suspension environment of the LCB material inside the reactor so proper mixture can be achieved, enhancing molecules contact and collision, leading to an increase in the hydrolysis reaction rate [63]. The advantages and disadvantages of this type of pretreatment are present in section 2.4.1.

2.4 Pretreatment review and applications

To succeed and accomplish the most complete possible fractionation of this raw-material in their main components it is necessary to optimize the pretreatment step accordingly to the process objective, allowing the reduction of the natural resistance to deconstruction and therefore enabling components recovery in the DSP, not only to reduce the resultant component into fermentable monomeric sugars but also recover the resultant well structured polymers that are, in themselves, high-value products. [60] Since biomass feedstock physicochemical characteristics differ from type of biomass to another it is usually necessary to use different combinations of pretreatments regarding the source in order to achieve effectiveness. which means, what is efficient for one type of LCB might not be for another one [1].

When establishing a pretreatment protocol it merits attention that this step has also major effects in the overall conversion of the biomass, influencing both the Upstream Processing (USP) and the DSP. In the USP the pretreatment definition might be imposed by the feedstock or vice versa, as pretreatment efficiency is different for each biomass. The choice of the pretreatmens/feedstock combination also influences biomass harvesting and storage as well as the temperature and pressure conditions that will be used during pretreatment [1, 45]. This step also influences the enzyme loading and the enzymatic hydrolysis rate. As for the DSP not only hemicellulose sugars are released from the biomass structure to the liquid medium but also part of the compounds derived from lignin, extractives and other biomass constituents are present. This components, as well as their content in the liquid fraction can have significant impact on the final product concentration and purification, on subsequent fermentation steps - even leading to cellular growth inhibition, on the waste treatment demands and on the fraction of water that can be recycled [45, 49].

As explained, the existing pretreatment technologies present some obstacles to overcome, including an insufficient separation of cellulose and lignin, formation of compounds that inhibit fermentation, high energy demands and/or use of chemicals, and considerable production of wastes [8, 66]. As described in the beginning of chapter 1, although a large number of technologies have already been proposed for biomass pretreatment due to intensive research and development investment [5], the choice of the best technology is not an easy task to preform since it depends largely on the objective of the pretreatment (desired products), the type of biomass that will be used, and its composition. For the development of an effective, economically viable, and sustainable pretreatment technology, understanding the structure of biomass, selecting the most suitable method for target components, and operating under optimal conditions are necessary requirements in order to proceed. [1].

Since LCB is an important source in terms of sustainability and price for new bioactive substances, this work will report the extraction profile of valuable compounds from one type of the many available, but underutilized resources, as it is wheat straw. This material is also a potent source of nutrients

section 2.2.2 for microbial cultivations for the production of bioactive substances. Previous studies have stated that, in order to be successful when using this type of biomass as substrate for cellular growth, metabolites production, it is mandatory to make sure that (1) sugar concentration meets the necessary values for further utilization; (2) inhibitory compounds, present in the biomass source and/or formed during extraction procedures, are still below inhibitory concentration thresholds; [67, 68] . Nevertheless, since this work is based on the paradigm of a biomass refinery [69], it is worthily to pursuit further the study of substances with possible valorization that might be within the mixture after fractioning and have not been extensively studied previously, namely, extracted lignin concentration in the liquid stream after a LHW procedure. In essence, the goal of this section is to evaluate by looking to previously published work, the performance of a wide branch of different extraction technologies and operational conditions using an abundant, inexpensive and sustainable resource and study if it is possible to obtain bioproducts profile (sugars, degradation products and lignin) that can be put to use in a biorefinery valorization context, always taking into account sustainability, cost-effectiveness and environmental footprint.

2.4.1 Pretreatment criterium considerations

In this section, it is stated the strategies used in previous works on their approach to collapse the rigid and recalcitrant LCB structure and and what factors need to be considered regarding their influence on the final product substances concentration profile.

Previously studies have demonstrated how some critical influencing and interconnected factors that can change the final pretreated mixture, depending basically on:

1. Thermodynamic harshness towards the raw-material (R_0);
2. Reagents used in the process;
3. Equipment and operational limitations.

Following this rational, thermohydraulic pretreatment methods, such as LHW, are characterised by using high temperatures and pressures, and so high severity factor values, in order to make the LCB main components accessible for hydrolysis and recovery (see section 2.3.2). This type of technologies have proven to be effective by several authors when the goal is to obtain hemicellulosic sugars [25, 51] without disrupting, either the cellulose matrix, a valuable product itself, as it is explained in section 2.2.2.1, nor lignin structure, aiming for an upstream extraction, in a biorefiney recovering step, with a more effective method specifically for lignin recovery (see section 2.3.3). Gullón et al. (2010) [70] and Liu et al. (2015) [71] stated that if a LHW fractioning pretreatment is applied, the cellulose and lignin contents in the remaining solids increase, which means, the remaining substance of interest, hemicellulose, is being transferred to the liquid fraction via solubilization. Ruiz et al. (2012) [72] work goes even

further and specifies autohydrolysis as the solubilization of hemicellulose and organosolv as solubilization of lignin for the pretreated solid' stream that was obtained for each fractioning step. It is worthily to remark that previous literature have not produced enough, significant and explicit studies on lignin concentration when comparing to sugars concentration profiles, when changes in the operational conditions R_0 take place. With more harsh reaction conditions, the lignin can suffer disintegration and lose value as final product. So when a biorefinery mindset is applied, the aim is to minimize the lignin losses in the upstream fractioning steps in order to ensure that, downstream, the lignin content can be extracted as a hole value-added product, for example, through an organosolv extraction procedure.

Regarding on how operational conditions affect the liquors substances concentration profiles using LHW, Carvalheiro et al. (2004) [73] studied the influence of temperature and holing time variations regarding sugars and degradation products. Using brewery's spent grain as raw material, it was observed an increase on degradation products concentration with the use of more severe operational conditions, or in other view, with the increase of the R_0 value. Looking closely to the sugars concentration it is clear that there is an increase on this variable value until a certain holding time, for each of the holding temperatures that were evaluated (150, 170 and 190 ℃), and then, when it reaches the maximum, the concentration starts to decrease and, in the limit, might tend tor wards the null value. This indicates, with accuracy, that there is a limit in the operational conditions harshness (R_0) and when this value is exceeded, the reaction's equilibrium between sugar production and degradation products formation (sugar decay) tends towards the formation of this inhibitory substances. Similarly to this case, Michelin et al. (2016a) [74], using the conditions of 180 ℃ and 200 ℃ (holding time of 30 min for both experiments) for sugarcane bagasse pretreatment shown an increase in furfural concentration from 0.5 g/L to 4.8 g/L, respectively, exceeding its concentration threshold for yeast fermentation. This is also explicit in another project, by Michelin et al. (2016) [25] in which the authors present a pretreatment of wheat straw that it is detected low levels of sugar degradation products concentration (0.65 g/L of acetic acid, 0.07 g/L of HMF and 0.12 g/L of furfural), which means that, using LHW with the operational conditions of 190 ℃ for 30 min of holding time, this values are within the thresholds of degradation products concentration stated by Palmqvist et al. (1999) [75] and Sanchez & Bautista (1988) [76]. In the first case, this work reports that in the case of the bakers' yeast the fermentation is not significantly affected by acetic acid and furfural up to a concentration of 10 g/L and 2 g/L, respectively. Regarding HMF, Sanchez & Bautista (1988) [76] reported that furfural begins to affect *Saccharomyces cerevisae* at the concentration of 1.5 g/L with total inhibition at 2 g/L and the concentration of HMF seems not to inhibit the yeast metabolism (CO_2 production unchanged) aldo an increase in the latency period was observed. The work of Beisl et al. (2019) [77], using LHW for sugar production, reported a monomeric C5 and C6 sugar concentration, after a concentration step, of 1.0 g/L and 6.0 g/L, respectively, and a total sugar concentration , again for C5 and C6, of 3.0 g/L and 10.7 g/L respectively, with HMF and furfural concentrations below the equip-

ment detection limit and an acetic acid concentration of 1.84 g/L. This last concentration was proven to be inhibitory of *Sacharomyces acidocaldarius* cellular growth, even after a distillation and dilution steps, which gave a final acetic acid concentration of 0.064 g/L. Only treatment with activated carbon allowed this microorganism to grow, and even with the reduction of sugar concentrations due to the purification steps, the cellular growth profile with time mimicked the one used in a reference medium, during more than 2 days of fermentation. On the other hand, taking the acetic acid enriched fraction (with a concentration of 3.1 g/L) of the distillation step and using it in the cultivation of cyanobacteria even allowed a higher cellular growth than the one using the reference medium. On this work perspective, despite the successfully achieved sugars production originated from LCB pretreatment using LHW, the application of the remaining liquid fraction is highly dependent on the microorganism.

Summarizing, autohydrolysis has many technological advantages as compared to other pretreatment processes, and this includes: (1) inhibitory products concentration below the threshold for fermentations; (2) allows oligosaccharides and monosaccharides to be extracted and solubilized which can be used for in the pharmaceutical and food industries; (3) high solid recovery, rich in cellulose content and high-quality lignin that can be further more valorized in the biomass refinery.

To continue the pursuit of a weighted choice on which pretreatment can be applied there is always the need for reagents properties consideration, not only regarding its effectiveness towards the objective of biomass fractioning but also considering the operational costs that come with its use, which means price and equipment wear and tear.

The use of acids as for biomass pretreatment, for example, has been studied and applied since the 19_{th} century as an effective way of LCB matrix deconstructing, being effective on hemicellulosic and cellulosic sugars recovering, as it is stated in the works of Carvalheiro et al. (2008) [12] and Mussatto (2016) [46]. Carvalheiro et al. [12] stated, in 2008, that the production of degradation products originated from sugar degradation is not significant. Nevertheless, Marzialetti et al. (2008) [78], published that the concentration of degradation products only reaches the threshold of inhibitory concentration values if the operation conditions harshness towards the raw-material increase significantly, which means, above 200 ºC and a holding time of 60 min, specifically for this work. It appears that Mussatto [46] is closest to this opinion that Carvalheiro et al. [12]. Moderate operational conditions, as it is presented in the work of Guerra-Rodriguez et al. (2012) [47], might still achieve the goal of sugar production (25.5 g/L of fermentable sugars) and maintain low degradation products concentration (0.9 g/L of furfural-HMF and 2.3 g/L of acetic acid) within the threshold for microorganism fermentation and cellular growth described before by Palmqvist et al. (1999) [75] and Sanchez & Bautista (1988) [76]. Considering both published concentration thresholds, it is correct to state that the remaining sugar liquors that were produced by Guerra-Rodriguez et al. (2012) [47] could be used in *S. cerevisae* fermentation processes. However, the major setback when considering acid hydrolysis as a pretreatment method for this dissertation work

is the non-specificity of the acid reaction between celluose and hemicellulose, namely when using concentrated acids [46]. In the work of Duarte et al. (2009) [79] it is well shown that using acid hydrolyses methods, even with diluted concentrations of sufuric acid (4% H_2SO_4), it can be achieved a glucose concentration around 1.31 and 1.43 g/L for a reaction time of 30 and 60 min, respectively, even after a first autohydrolysis step that was able to recover 0.18 g/L of glucose. This differences in glucose concentration between those two consecutive steps gives the assurance that acid pretreatment goes further deep in the LCB matrix for glucose extraction, already disrupting the cellulose complex, which is, as stated before, a valuable product itself, and with no interest on being destroyed with severe acidic reactions. Similarly, concentrated acid will not only react to greater extent towards cellulosic material, but also behave in the same way when facing the lignin structure. The Klason lignin assessment uses this property to assess the lignin content (acid soluble, acid insoluble and total lignin concentration) within the solid fraction. This based on the standard protocol of two-stage acid hydrolyses that use both concentrated and diluted acid as reagents [46, 47].

From all these previously presented premises it follows that acid hydrolysis, despite of all the intrinsic advantages of the method itself, might not be able to sustain a specific sugar extraction from the hemicellulose matrix. The existence itself of a protocol that uses the acidic reaction proprieties to deconstruct the lignin structure for *a posteriori* assessments makes the use of this fractioning method an unviable solution for specific hemicellulosic sugars extractions. If the goal is to have the most specific extraction possible in this branch of the biomass refinery, one of the major objectives needs to be aim as much as possible for the LCB component that it is interesting to produce sugars from, which means, focus the reaction only on the hemicellulose structure while maintaining as much as possible the other valuable components structure, namely, cellulose and lignin. Technical limitations regarding the corrosion of the reaction equipment is also needed to be taken into consideration, even when using diluted acids to preform the hydrolysis step [46], as it was referenced in section 2.3.1. It is expected to have an increase in maintenance costs if this type of reagents is used for LCB fractioning in stainless steel reactors, for example.

Taking now in consideration lignin as a substance of interest, it is well described by previous authors that one of the main strategies for LCB delignification is applying organosolv pretreatment steps. The use of organic solvents, explained in section 2.3.3, not only hydrolysis the hemicellulose fraction of the biomass but also allows lignin solubilization and removal with maintenance of the lignin structure. When the solvent's action is combined with the use of a catalyst (e.g.: acid) a more selective delignification is achieved and the final lignin product itself is less degraded which adds value and appliance to this component for further technical applications [12]. Weinwurm et al. (2012) [58] compared the changes in concentration profiles between the pretreatments of LHW and ethanol organosolv using wheat straw as raw-material for sugar and lignin extraction. The authors found that the use of the ethanol-water

mixture, instead of just water, for the same temperature and time of holding (180℃ and 60 min), allowed the increase of lignin removal from the biomass. When looking at the percentage of solubilized lignin for both processes, when using a mixture of 50 % etanol (w/w) this percentage value varies between 18% and 30% while using only water this percentage is fixed at 6%. Regarding sugars, organosolv proved to be less effective in sugar solubilization than autohydrolysis, which means, the more ethanol present in the reaction media the less sugar content was reached. Taking the example of glucose, the concentration found in the liquid phase decrease from 1.5% using the hydrothermal treatment to only 0.1 % when organosolv is applied. Notwithstanding, the formation of fermentation inhibitors could not be detected using ethanol as solvent.

As described in section 2.3.3, the reagents used in this type of fractioning procedure present a more expensive cost than the ones used in LHW or acid hydrolysis. Even more, when considering the impact of organic solvents usage, not only in an environmental friendly point of view but in terms of safe handling, there is the need of taking additional wide safety measures to ensure minimal exposure torwards the operator and residual impact in the nature [21]. Moreover, organosolv is considered by Wang et al. (2007) [80] to have the highest environmental impact of the main lignin extraction processes (kraft, sulfite, soda and organosolv procedures) per kg of lignin-based products, which reinforces the idea previously described. One of the approaches used to minimize solvent waste is to apply recovery techniques, such as direct reagent draining from the reactor, re-fluxing the evaporated stream followed by a condensation step for solvent recycling [44]. This process solution not only treats the waste stream, protecting the environment, but also reduces the amount of fresh reagent in the inlet stream of the overall process (e.g.: in a biomass refinery [81]). On the other hand, biopretreatments, for example, enzymatic treatments, can become a widely used biotechnology in the biomass refineries context. This type of fractioning procedures have the advantage of combining the previously described advantages of compared to other pretreatment processes, and this includes: (1) high sugar yields and specificity for hemicellulosic and cellulosic biomass; (2) no direct degradation products formation; (3) no lignin degradation; (4) no harmful nor pollutant residue formation; (5) applied to a wide spectrum of raw-materials; (6) already studied and dimensioned for pilot scale. The work of Zhang et al. (2013a) [82] shows that a combined enzymatic treatment with other chemical-based procedures (in this case, diluted acid hydrolysis) can result in a maximum glucose yields of 93 %, compared to a yield a maximum yield of 22% for the same type of sugar using only hydrochloric acid. However, the enzymes price for high catalytic purity and specificity, demanded by the pharmaceutical industries, is presented as the major setback for medium-scale bioindustries applications, as it is stated in section 2.3.4.

Summarizing all of this considerations, it was possible to construct a decision table, table 2.2, that allows the choosing of the more suitable pretreatment for this work's objective, which means, the fractioning method that allows the production of enough lignocellulosic sugars for further applications hav-

ing degradation products concentrations within the threshold for fermentations and cellular growth while maintaining the lignin structure intact, as much as possible, for further extractions upstream the biomass refinery. As tiebreaker factors, it will be chosen the less pollutant and cheapest pretreatment from all the available technologies so the goal of sustainability can be achieved together with the driving force for a greener and cleaner bioindustry. As it was stated in this dissertation title, the pretreatment that was chosen to pursuit this goals was the autohydrolysis fractioning method.

Table 2.2: Pretreatment's decision table.
LHW: Liquid Hot Water; DAH: Diluted Acid Hydrolysis; CAH: Concentrated Acid Hydrolysis; OS: Organosolv; Enz: Enzymatic Treatment;
(√ √): highly advantageous; (√): advantageous; (-): neither advantageous nor disadvantageous; (X): disadvantageous; (X X) highly disadvantageous.

Type of Pretreatment	LHW	DAH	CAH	OS	Enz
Sugar yield	√ √	√ √	√ √	√	√ √
Inhibitor Formation	X	X	X	√ √	√ √
Delignification	√	√	√	√ √	X X
Residue Formation	√	X	X	√	√ √
Chemicals used	√ √	X	X	X	√ √
Investment Cost	√	-	X	X	X X
Operational Cost	√	√	-	X	X X
Substrate Range	√	√	√ √	√	√ √
Proven at Pilot Scale	√ √	√ √	√ √	√ √	√ √

3

Materials and Methods

Contents

3.1 Wheat straw as lignocellulosic feedstock

The LCB used in this work was wheat straw harvested in 2019 in the region of Margarethen am Moos, state of Lower Austria and stored at TU Wien lab, at room temperature in a closed polyethylene terephthalate (PET) box away from direct light. The particle size was reduced in a cutting mill, equipped with a 2 mm mesh, before pretreatment.

3.1.1 Wheat straw characterization

This wheat straw was previously described by Serna-Loaiza et al. (2020) [40], following the National Renewable Energy Laboratory (NREL) protocol [83] for the determination of structural carbohydrates, lignin, extractives and ash in biomass. This authors also determined this raw-material moisture content, useful not only, to determine the components weight percentage in a wet basis, but also for sample preparation (see section 4.1). The moisture content was assessed using Sartorius® moisture analyser model MA 150. The results are present in the following table 3.1:

Table 3.1: Wheat straw characterization regarding structural carbohydrates, lignin, extractives and ash in biomass. The left table represents the experimental values for the dry weight of each component. According to the NREL protocol, described by Sluiter et al. (2012), since the sum of all weight percentages (%wt) is above 97 %, it can be assumed a 100 % dry basis. The values on this last basis are present in the center table. The right table represents the weight percentage values for the wheat straw on a wet basis, for each component, having in consideration the moisture content.

Component	Weight percentage Exp. values (%wt)	Error (%)
Arabinan	2.13 ± 0.16	7.5
Galactan	0.67 ± 0.01	1.5
Glucan	35.31 ± 2.14	6.1
Xylan	21.94 ± 0.60	2.7
Mannan	0.72 ± 0.05	6.9
Lignin	17.35 ± 0.99	5.7
Extractives	20.45 ± 1.26	6.2
Ash	1.09 ± 0.07	6.4
Total (Dry Basis)	99.66	-

Weight percentage dry basis (%wt)	Error (%)
2.14 ± 0.16	7.5
0.67 ± 0.01	1.5
35.43 ± 2.15	6.1
22.01 ± 0.60	2.7
0.72 ± 0.05	6.9
17.41 ± 0.99	5.7
20.52 ± 1.26	6.2
1.09 ± 0.07	6.4
100.00	-

Weight percentage Wet Basis (%wt)	Error (%)
1.98 ± 0.15	7.5
0.63 ± 0.01	1.5
32.89 ± 1.99	6.1
20.44 ± 0.56	2.7
0.67 ± 0.05	6.9
16.16 ± 0.92	5.7
19.06 ± 1.17	6.2
1.01 ± 0.07	6.4
92.84	-
7.16 ± 0.07	1.0
100.00	-

It is worth to remark that the errors for all the components are always below 8% so it can be stated that all the results are statistically valid.

3.2 Fractioning of wheat straw raw-material

LHW, or autohydrolysis, was carried out at laboratory scale in a stainless steel high pressure autoclave STR (Zirbus, HAD 9/16, Bad Grund, Germany) provided by TU Wien (Institute of Chemical, Environmental and Bioscience Engineering, Vienna, Austria). The reactor has a working volume of 1 L and maximum temperature and pressure of 250℃ and 60 bar, respectively. The autoclave is equipped with two external mantles for heat exchange, one electric for heating and other connected to the tap water grid for cooling. The temperature and pressure where controlled every second (Δt for severity factor computing). The stirrer used was a single turbine impeller with 4 vertical blades with a left sloped cut on the edge of each of them for better mixture. Each run was performed with a clockwise rotation of 150 rpm.

The LSR was 11 grams of type 1 water to 1 gram of dry wheat straw, for every run. From this point forward, type 1 water (water for injection) will be only described as water or H_2O.

The experiments were carried out using isothermal conditions (see section 2.3.2) in which the reactor was set to be heated until it reaches the temperatures of 160, 180 and 200 ˚C and held at that set point for 30, 60 and 90 minutes for each run. This 9 combinations of experimental settings were made in triplicate, making the total number of 27 experiments. Once the holding time was achieved, the reactor was then cooled down until the inside product enters thermal balance with the cooling stream. The heating profiles considering heating, holding and cooling phases were plotted. After the cooling step, the reactor was opened and the remaining liquid and solid phases were recovered. The pretreated mixture was weighted for further mass balances assessments. The separation between these two phases was conducted using a regular stockings nylon membrane (Clever ®) where the solids were putted inside the bag-like sock and separated using a hydraulic press model HAPA-Presse HPH 2.5 with work pressure up to 150 bar to avoid the filtering membrane bag disruption. The pretreated solid and liquid fractions were weighted for further mass balances assessments. The remaining liquid phase was centrifuged using the Sigma 4K15 ultracentrifuge (Linder Labortechnik ®) at 14,000 rpm for 20 min at the set up temperature of 20 ºC for the supernatant recovery. The remaining pellet was discharged.

The remaining liquid fraction was stored in a 500 mL Schott ® flask at 4 ºC for future use and assessment. The supernatant liquid was weighted for further mass balances assessments. On the other hand the remaining solid fraction, after the drying step (see section 3.3.1), was stored in a closed zip plastic bag at room temperature. All the weighting steps were performed using the Sartorius ® scale model GP 4102, with an maximum error of $\pm$ 0.01 g.

3.3 Analytics

The following section describes the analytical methods used in the characterization of the wheat straw raw-material after the respective fractioning, as well as the equipment used to perform the analyses.

3.3.1 Remaining fractions moisture and solid content

A sample of both remaining solid and liquid phases were then assessed in terms of moisture and solid content, respectively. In order to do this, glass vials were used, previously dried and tared in a drying oven VENTI-Line VWR ® at the set up temperature of 105˚C. The vials were cooled down in a desiccator and both samples from the two fractions were put inside the vials and the total weight was noted. After this step, both samples were dried over night in the same oven under the conditions described before. It was used a single sample for the liquid fraction solid content assessment and a duplicate for the moisture content in the remaining solids. In the following day, the samples were cooled down in the desiccator, as before, and weighted again. All the weighting steps were performed using an analytical scale KERN ® ABT 320-4NM, with a maximum error of $\pm$ 0.001 g.

3.3.2 Liquid fraction density

The density of the remaining liquid fraction was measured in a Mettler Toledo ® DE45 Delta Range Density Meter by injecting the desired liquid through the inlet capillary tube in order to get a continuous liquid inside the equipment, specifically, without any air bubbles. Three measurements were made it was assumed the mean value was used for computation purposes.

3.3.3 Mass balance and losses report

With the objective of assessing the mass losses between in the DSP steps it was measured the various weight of both solid and liquid fractions, before and after each step. All the weighting procedures were performed using the Sartorius ® scale model GP 4102, with an maximum error of $\pm$ 0.01 g.

3.3.4 Ash content

The determination of the ash content follows the NREL protocol [83]. For most of the experiments, the ash content weight that was obtained was always bellow the equipment determination error (data not shown). The only experiments that showed positive values indicated an ash content in the solid samples of approximately 2 %wt and of 0.06 %wt in the obtained extracts. This value was considered constant for all the extractions and equal to 1.09 $\pm$ 0.07 %wt.

3.3.5 Lignin content in the liquid fraction

For the lignin quantification in the lignin fraction it was applied the NREL Protocol [84] based on a quantitative acid hydrolysis of the dry matter content of the extract. This protocol assess the Acid Soluble Lignin (ASL) and the Acid Insoluble Lignin (AIL) concentration for each trial. After drying enough volume of liquid fraction sample in the drying oven as described before (*vide* section 3.3.1) it should be weighted 300 $\pm$ 1 mg of the remaining solids in an autoclave resistant Pyrex ® tube. After adding also 5 ml of 72 % sulfuric acid (H_2SO_4) the sample was kept at 30 ℃ for 1h in a water-bath and stirred with a glass rod every 10 minutes. The sample was then diluted in order to get an (H_2SO_4) concentration of 4 % in the total sample volume and then this mixture was autoclaved at 121 ℃ for 1h. After this, the samples were cooled down to room temperature and vacuum-filtered. The filtration was performed with a Büchner funnel and filter paper Sartorius ® grade 388, previously weighted, and a diameter of 110 mm. The first liquid fraction was collected and used for the determination of the ASL. Then, the tube was washed with water to collect all the solid in the filter paper. To obtain the value of ASL concentration, the permeate was then analysed in the spectrophotometer, at 205 nm, and diluted accordingly to be in the range of absorbance between the values of 0.6 and 1. Considering that the remaining solids in the retentate correspond to the AIL value, the paper filter and its content was dried over night as described before (*vide* section 3.3.1) and, when it was back at room temperature, the final weight was noted and the concentration value assessed.

3.3.6 Degradation products in the liquid fraction

Furfural, HMF, and acetic acid concentrations were determined accordingly to the NREL protocol [84] using High Performance Liquid Chromatography (HPLC) (LC-20A HPLC system, model SPD-M20A IVDD, Shimadzu, Japan) by UV and RI detection with a Shodex SH1011 analytic column at 40 ℃ with 0.005 M (H_2SO_4) as mobile phase and a flow rate of 0.6 mL/min. An ultra-centrifugation step was made, as described before in section 3.2, prior to sample insertion, so the does not get clogged. At least, 20 µL of the remaining supernatant volume was taken, transferred to the correspondent equipment glass vials and placed in the respective HPLC sample tray. A stock solution of the measured degradation products was prepared and diluted accordingly (appendix A.5.1). These standards were used to calculate a calibration curve, from which the concentration of the analyzed samples was determined.

3.3.7 Sugar concentration in the liquid fraction

During the LCB pretreatment, carbohydrates, which make up the most of the biomass content, are released to the liquid fraction in the form of soluble sugars. These polysaccharides consist mainly of glucose, xylose, arabinose, galactose and mannose. In order to assess how the sugar concentration

behaves with changes in the LHW fraccioning conditions, when pretreating LCB, the sugar concentrations within the LHW outlet liquid fraction were quantified by subjecting all arrays to a hydrolyzed and non-hydrolyzed sugars assessment, that provides information about the amount of total and monomeric sugars present in the solution, respectively. Monomeric sugars were analyzed using HPAEC-PAD (ICS-5000, Thermo Scientific, USA) with deionized water as eluent. Oligomeric sugars were hydrolyzed (diluted sulfuric acid) at 120 ℃ and analyzed as monomers. A sugar recovery standard was used to account for losses. For monomeric sugars, a 1 mL sample was taken from each trial liquid fraction, diluted with a factor of 1:20, and then put to analyse in the High Performance Anion Exchange Chromatography (HPAEC) equipment. The hydrolization of the remaining oligomers must be made for the total sugar's concentration assessment. It was added 1 mL of a 4% (H_2SO_4) solution to 0.5 mL of each arrayand then make up the volume with water to 10 mL, obtaining a 1:20 dilution factor. An analytical set of Sugar Standard Solutions (SSS) was prepared for calibration and control of the area of integration *vs* sugar concentration behavior. Nevertheless, a proper assessment and correction of losses due to decomposition of sugars during dilute acid hydrolysis must be made. Therefore, a set of Sugar Recovery Standard (SRS) was also prepared. Both solutions preparation procedure - for SSS and SRS - are described in appendix A.5.2. All of this mixtures must be done in autoclave resistant Pyrex ® vials with their respective lid in order to avoid liquid transfer losses or leaks. The samples, the SSS and the SRS, were then put to react in an autoclave at 121 ℃ for 1h, similar to the procedure described in section 3.3.5. After cooling down, it followed another dilution step of 1:10, in order to make the final dilution factor 1:200, and an ultra-centrifugation step by an Eppendorf ® centrifuge model 5418 R for 20 min at 14 500 rpm with the goal of avoiding HPAEC equipment capillary lines from clogging with precipitated lignin that might be present in the liquid sample. The supernatant is then transferred to proper equipment glass vials and putted in the respective HPAEC equipment tray for sugar determination. For both assessments, if the obtained concentration picks were not within the range of the standards used for calibration, the samples were diluted to fulfil this criterium, accordingly to the NREL protocol [84].

3.3.8 Severity factor

The Severity factor, proposed by Overend and Chornet (1987) [53], can be computed using the following mathematical expression:

$$R_0 = \int_0^{t_{op}} exp\left(\frac{T(t) - T_{ref}}{\omega}\right) dt \tag{3.1}$$

In which T(t), in Celsius, gives the temperature profile with the reaction time, t, in minutes and T_{ref}, in Celsius, is the reference temperature. Previous authors have fixed the value of 100 ℃ for this reference temperature. In order to be able to compare and discuss the obtained results with them, in this work,

this value was also used in all the calculations. The time of operation, t_{op} (min) is also considered to be the time for which the reactional mixture is above T_{ref}.

The empirical parameter ω, is commonly assigned to the value of 14.75 (dimensionless), assuming an overall reaction following first-order kinetics and Arrhenius relation of temperature [85]. This parameter is given by the equation 3.2:

$$\omega = \frac{T_f^2 R}{E_a} \tag{3.2}$$

Being: T_f, the floor temperature, meaning, the temperature in the middle of the range of experimental conditions; R as universal gas constant; and E_a as the energy of activation.

By the reasons described before, this parameter value will also be considered equal to the one most frequently used in literature, enabling discussion and comparison with previous works.

The severity factor was calculated using the trapezoidal rule, described by:

$$\int_a^b f(t)dt \approx (b-a) \cdot \frac{f(a)+f(b)}{2} \tag{3.3}$$

When eq. (3.3) is applied to the severity factor, meaning to eq. (3.1), it gives the final expression present in eq. (3.4). This expression was computed using Excel ® as data processing tool. As stated in section 3.2, the considered time interval, the Δt, was one second (for computational purposes it was considered 1/60 min $\approx$ 0.016(6) min).

$$\int_0^{t_{op}} f(t)dt \approx \sum_{i=0}^{t_{op}} \left(\Delta t \cdot \frac{exp\left(\frac{T_t - T_{ref}}{\omega}\right) + exp\left(\frac{T_{t+\Delta t} - T_{ref}}{\omega}\right)}{2} \right) \tag{3.4}$$

3.3.9 Empirical modelling

Several models based on the experimental data were tried making use of the Microsoft ® Excel program for Mac version 16.42, using the function Linest adapted to non-linear models. The degradation products, lignin and sugar concentrations were modeled, having in consideration the R_0 given by the operation conditions harshness towards the raw-material. However, due to the profile behavior of sugars and lignin, combined with a small number of data points to be modeled, polynomial models, represented by the general eq. (3.5), were the only ones that could reproduce the experimental data for this components with some goodness of fit.

$$y(x) = a_n x^n + a_{n-1} x^{n-1} + ... + a_1 x + a_0 \tag{3.5}$$

This polynomial model is sustained by the mathematical theorem stating that a set of data is always described by the eq. (3.5) for a n value sufficiently high.

Regarding the models used to describe the three degradation products of this pretreatment step are

one-phase association, represented by eq. (3.6), and allosteric sigmoidal, decribed by eq. (3.7), in the following equations:

$$y(R_0) = y_0 + (Plateau - y_0) \cdot (1 - exp(-K \cdot R_0)) \tag{3.6}$$

Where y_0 is the y value when R_0 is null and it has the same units as $y(R_0)$; $Plateau$ is the y value at infinite R_0, expressed in the same units as $y(R_0)$ (in this case, concentration units) and; K is the rate constant, dimensionless.

$$y(R_0) = \frac{a \cdot (R_0)^h}{(b + (R_0)^h)} \tag{3.7}$$

Where a is the maximum product concentration, in the same units as $y(R_0)$. It is the highest given product concentration extrapolated to very high severity factor, and therefore, is almost always higher than any measured for a given experiment. The variable b equals $(K_{half})^h$, being K_{half} the severity factor that produces a half-maximal degradation product concentration and; h is the hill slope.

The one-phase association equation describes the pseudo-first order association kinetics of the interaction between a ligand and its receptor. During each severity factor interval a certain fraction of the substrate reacts, and is lost. As the severity increases, fewer sugar concentration is available for degradation and the curve levels off. In the case of the allosteric sigmoidal, it is implicit an alteration of the degradation activity induced by a different molecule, a catalyst (probably acetic acid). In this cooperative behavior, the probability that the remaining sugar substrate bonds will hydrolyse increases, leading to the conversion of sugar degradation into by products, which will provoke the rise on this molecule concentration, until the sugar concentration available for degradation approximates the null value and the curve levels off. It is known that an increase in the acetic acid concentration facilitates the sugar hydrolysis making this process autohydrolyzed [25]. As testing criterium for all this assumptions, the models goodness of prediction, will be evaluated taking into account the relative percent deviation (RPD) between the values computed by the two models and the experimental data. The goodness of fit will also be considered by evaluating the correlation coefficient value (R^2).

3.3.10 Yield and conversion assessment

The values for each structural carbohydrate component and lignin weight percentage, present in table 3.1, are useful to determine the yield and conversion, respectively, for each fixed operational condition, calculated by the following expressions:

$$Y_{P/S} \left(\frac{g\ monomeric\ sugar}{g\ carbohydrate\ substrate} \right) = \frac{monomeric\ sugar\ (\%wt) \times Corr}{Carbohydrate\ substrate\ (\%wt)} \tag{3.8}$$

$$X_{Sl/L}\left(\frac{g\ solubilized\ lignin}{g\ lignin\ source}\right) = \frac{Solubilized\ lignin\ (\%wt)}{Lignin\ (\%wt)} \tag{3.9}$$

Where P is defined as each monomeric sugar that is produced having their respective structural carbohydrate as substrate, S. This production occurs by the hydorlization of the polymeric sugar chains which result in addition of a water molecule into the produced sugar monomer. This is taken into consideration, when calculating the yield of production relatively to the substrate available in the raw-material, by the $Corr$ parameter, which is the anhydro weight correction of 0.88 (or 132/150) for C5 sugars (xylose and arabinose) and a correction of 0.90 (or 162/180) for C6 sugars (glucose, galactose, and mannose) in order to calculate the concentration of the monomeric sugars in an anhydro base, following the NREL protocol [83]. In the case of lignin, it is applied the eq. (3.9), for the amount of structural lignin, L, that is converted into solubilized lignin, Sl.

4

Results and Discussion

Contents

4.1 Dry matter content and sample preparation

It is mandatory to assess the LCB dry matter content so that a proper LSR can be obtained when preparing the mixture of water and raw-material, prior to the transfer into the reactor (see section 3.2). The value of 0.9284 g of dry wheat straw/ g of wet wheat straw was obtained. The water content in the wheat straw needed to be taken into consideration to have a LSR of 11 grams of water to 1 gram of dry wheat straw. First, it was established by the project parties a fixed amount of 40 g of dry wheat straw and 440 g of total water within the mixture. But, with this mass values, there was the need of two ultracentrifugation cycles, making the process more time consuming and the reaction mixture tended to overflow the mixer holding plate, which increased the losses in the sample collection step from the reactor. With this empirical feedback, after the first trials, the amount was changed to 30 g of dry wheat straw and 330 g of total water. The deviation between the replicates in this 2 first trials - with the operation conditions of: 200℃ and 30 min of holding time and 160℃ and 30 min holding time - was neglected since they were within the acceptable range of error (considered by the parties to be a maximum of 8%) and the LSR was maintained. All the mass balance equations used to obtain the other variables are described in appendix A: for wet matter (g) see eq. (A.1); for water content (g) see eq. (A.2) and eq. (A.3) and; for water to be added (g) see eq. (A.4) and eq. (A.5). The respective values needed to describe the sample preparation before the reaction are described in table 4.1.

Table 4.1: Wheat straw sample mixture preparation for autohydrolysis fractioning. The dry matter (g) refers to the wheat straw, and LSR to liquid-to-solid ratio.

LSR	Dry matter (g)	Total water (g)	Wet matter (g)	Water content (g)	Water to be added (g)
11:1	40	440	43.0849	3.0849	436.92
11:1	30	330	32.3137	2.3137	327.69

The wheat straw dry content naturally changes if the raw-material source, storage, transportation or place of handling are different. As a matter of fact the LCB composition changes with the weather and the harvest season. So in order to normalize all these variables, if the case of a change in any of the parameters, the moisture content must be assessed again and new mass balance values prior to the fractioning should be noted. This explains why the moisture content values presented in literature are different from each other. For example, these changes are well illustrated when comparing LCB described by Carvalheiro et al. (2009) [86], that stated a 92% dry content in wheat straw from Élvas, Portugal, in 2009 with 2011 french wheat straw, with 89.4 % [87], from the Charent-Maritime region, described by Pierre et al. (2011). More specifically, from Upper Austria federal state, Austria, Eisenhuber et al. (2013a) and Heoke Kahr et al. (2013) work is based on a wheat straw dry content of 91 % [88,89].

4.2 Autohydrolysis fractioning conditions assessment

As it was described in section 3.2 of the previous chapter, the mixture water/wheat straw was subjected to a range of temperatures and pressures, for a specific holding time at a certain set-up temperature, so the raw-material hydrolysis can be achieved and studied.

Since the reactor was equipped with a pressure and temperature sensor-metter it was possible to register, and plot, the temperature and pressure profile with a time-lag of one second. This temperature and pressure profiles give the feedback information on how the runs are being reproduced during the 27 trials (3 set temperatures, 3 set holding times made in triplicates), so the changes observed in this profiles are only due to operations conditions variation and not because of the equipment malfunction or mishandling. The evaluation criterium used to state if there was a major deviation between triplicates, was the deviation between the values of severity factor (R_0). As shown in fig. 4.1 and table 4.2, the temperature profile behavior for a given fixed operational parameter was not severely affect, nor the R_0 values deviations between the triplicates, since they were always within the acceptable error range of 8%.

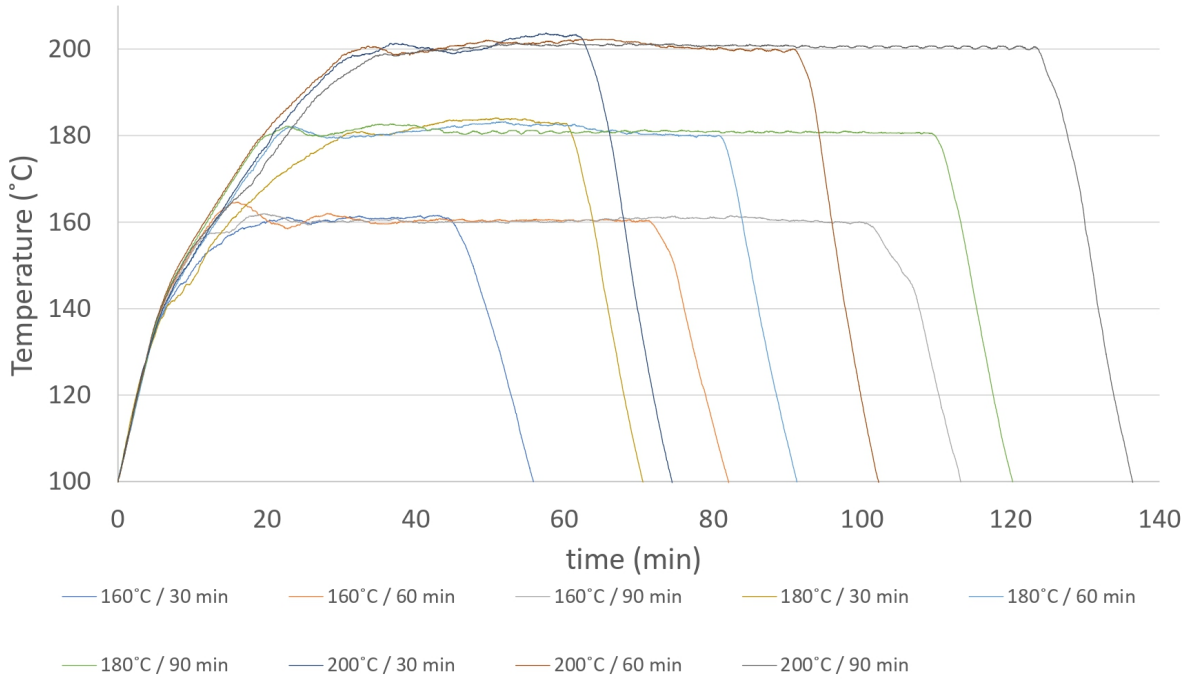

Figure 4.1: Inner product temperature profiles with the time of operation for a given set of holding conditions (temperature and time).

By the observation of fig. 4.1 it can be identified a similar experimental behavior between conditions, in terms of the heating, holding and cooling phases. In the first step, the temperature presents a slightly logarithmic behavior when the mixture is heating up, characterized by a fast heating rate in the beginning - when the temperature gradient between the inside product and the heating jacket is higher - but, as long as the temperatures converge, as expected, heating rate decreased since this driving force of heat exchange decreases. When the inside product achieved the objective temperature it is observed an overshoot in its temperature provoked by the delay between the sensor and the actuator. Since this happened in the majority of the runs and, when it did happen, it was for a short period of time (maximum 8 min of overshoot for the 160 ˚C and 60 min holding time run), this can be considered as not interfering in the overall comparison and assessment of the remaining products.

Regarding the second phase, meaning when the temperature is meant to be fixed during a specific holding time, sinusoidal behavior of the temperature profile was observed. This is justified with the increase in the inside reactor pressure, leading the mixture temperature also increase with time, following the 1st law of thermodynamics, eq. (A.6). With this type of reactor equipment, there was the need of manual stabilization of the inner product by changing the heating jacket temperature, in order to overcome this unwanted inside temperature increase. Since it is non-automatized process, gross oscillations in the product temperature tend to occur (see fig. 4.1), but, once more, since they were well within the acceptable range of experimental error, this work was allowed to proceed.

The last phase of the reaction is characterized by a fast decrease in the product temperature by the cooling tap-water system. Often, this phase contribution is neglected in the literature [25,90,91] and just cited as a rapid cool down step.

The severity factor calculated values, as the real holding time for all the experimental conditions and their respective statistical treatment are present in table 4.2.

Table 4.2: Severity factor R_0 and real experimental holding time values with the respective of the standard deviation and percentage of statistical error.

Temperature (℃)	Aim hold time (min)	R_0	Err (%)	holding time (min)	Err (%)
160	30	2385.2 ± 90.1	3.8	30.7 ± 0.2	0.8
	60	4109.3 ± 24.2	0.6	61.0 ± 0.3	0.6
	90	5884.8 ± 143.1	2.4	90.2 ± 0.2	0.2
180	30	11131.5 ± 434.4	3.9	31.6 ± 0.6	1.9
	60	16525.3 ± 348.6	2.1	60.7 ± 0.3	0.5
	90	23754.2 ± 229.6	1.0	91.1 ± 0.6	0.7
200	30	39628.2 ± 3152.0	8.0	30.6 ± 0.4	1.2
	60	64763.9 ± 2010.5	3.1	60.5 ± 0.3	0.4
	90	93064.7 ± 3081.6	3.3	87.1 ± 4.2	4.9

It is worth to mention that there was some issues regarding the electricity supply (routine checks that required an electricity cut-off; fire alarm setting off) in two of the three replicate for the condition 200

˚C and 90 min holding time, which caused an increase in the standard deviation value for this condition and, consequently, the statistical error.

Raising in the inner reactor product temperature gives, necessarily, a higher severity factor value since it is described, in eq. (3.1), as an exponential of a temperature value with time, being this the dominant parameter in the equation. The analysis of the several phases contribution to the severity factor are present in table 4.3.

Table 4.3: Contribution of the heating,holding and cooling steps of the severity factor for all the operational conditions. All the values are the average of the triplicates for each set of temperatures and holding times.

Temperature (℃)	Aim hold time (min)	Heat/Total R_0 (%)	Hold/Total R_0 (%)	Cool/Total R_0 (%)
160	30	16%	79%	4%
	60	7%	91%	2%
	90	6%	92%	2%
180	30	21%	76%	3%
	60	8%	90%	2%
	90	5%	93%	2%
200	30	21%	76%	3%
	60	12%	86%	2%
	90	13%	86%	1%

Analysing now the heating contribution to the total R_0 there is the trend for a decrease in the first, for a fixed temperature, when the holding time increases its duration. On the other hand, the holding phase tends, generally, to increase when the holding time is higher. This two behaviors are expected since the severity factor integration, when its being computed at higher temperatures for more iterations, it corresponds to a higher value of the exponential term of this parameter. This assessment gives the perspective on how experimental errors, during different reaction phases, can affect the overall outcome of the study. Different studies, using non-isothermal conditions, state a higher contribution of the heating and cooling phase for the R_0 computation [92], which is expected due to the lack of an holding phase in that protocol. To compensate this, an higher objective temperature may be required to achieve LCB fractioning, which means a decrease in the temperature gradient between in the heating exchange process, originating a decrease of this phenomena driving force, increasing the heating contribution for the total severity factor value. However, how it is shown in table 4.3, the main contributing phase using isothermal conditions for the severity factor is the holding phase, which means that, the operational variables of this work will have a major influence if not controlled properly. As shown in fig. 4.1, it can be observed some oscillations in the temperature values in this phase. Also, in table 4.2, the holding time average, in general, has exceeded the aimed holding time (exception made for the 200℃ and 30 min conditions) even considering the extreme values of the error range. This is mainly due to the lack of automatization in the time and pressure control which gave rise to, not only an on/off manual control behavior with frequent oscillations around the temperature set-point, but also a delay when interrupting the heat transfer into the reactor and, consequently, delayed the start of the cooling system. As shown in

table 4.3, the cooling phase represents less than 5% of the overall R_0, which it explains why this phase is described in literature as not being significant in the final R_0 value.

4.3 Downstream processing

In this section it is shown how the pretreated water/wheat straw is treated after the autohydrolysis reaction in terms of the separation and recovery of the liquid and solid stream. More specifically, it was studied the density of the sugar solution, the moisture content of the solid fraction and the losses between the several process downstream steps and their influence in the outcome results.

4.3.1 Density assessment

The density values for all the severity conditions applied in this work are well described in the following table 4.4:

Table 4.4: Liquid fraction density for the different severity conditions.

R_0	Density (g/mL)			Error (%)
2385.2	1.004	$\pm$	0.0000	0.00
4109.3	1.005	$\pm$	0.0001	0.01
5884.8	1.007	$\pm$	0.0002	0.01
11131.5	1.009	$\pm$	0.0000	0.00
16525.3	1.009	$\pm$	0.0002	0.02
23754.2	1.008	$\pm$	0.0000	0.00
39628.2	1.006	$\pm$	0.0002	0.02
64763.9	1.005	$\pm$	0.0001	0.01
93064.7	1.005	$\pm$	0.0001	0.01

It is well described in this table that the density values for each condition are statistically valid, presenting errors always bellow 0.03%. A higher density value may indicate a higher concentration of components in solution, which can be confirmed further in this thesis.

4.3.2 Solid fraction moisture content

The moisture content gives us the information on how much liquid was still in the solid fraction since this liquid has components of interest dissolved within. although the reactor was stirred during all the operation, an ideal mixture could never be achieved, so, rough changes in this parameter might influence the concentration of soluble components in the remaining fractions. For example, since the concentration of sugars is higher close to the solid matrix, a less efficient filtration step might implicate an inferior sugar concentration in the liquid stream. The moisture content values are present in table 4.5.

Table 4.5: Moisture content determination assessment (%wt) for each severity conditions and its respective statistical error values.

R_0	Moisture content (%wt)			Error (%)
2385.2	58.35	$\pm$	2.42	4.2
4109.3	57.54	$\pm$	0.14	0.2
5884.8	52.17	$\pm$	2.09	4.0
11131.5	48.07	$\pm$	2.60	5.4
16525.3	55.88	$\pm$	0.75	1.3
23754.2	54.25	$\pm$	4.60	8.5
39628.2	57.41	$\pm$	1.13	2.0
64763.9	58.47	$\pm$	5.86	10
93064.7	57.52	$\pm$	2.42	4.2
All	53.50	$\pm$	10.8	20

This values present a general error of 20% which will be considered as non-affective of the final concentration result of the compounds to be analysed. Also, the moisture content error for each of the severity conditions, individually, never surpassed the value of 10 % which proves the triplicates reproducibility.

4.3.3 Mass losses

The mass losses are, also, good indicator of the process reproducibility because of the reasons described before in section 4.3.2. The loss of significant mass, liquid or solid, might implicate, as well, a loss on the compounds concentrations in their respective fractions. Since this biomass refinery project is aiming for an industrial setup, this complex will not have losses during the downstream procedures, which means that, the important criterium to analyse here is not if the losses are high but to assess if the losses are maintained stable during all the severity conditions, allowing the comparison and study of all the outcome results. The percentage of losses are present in table 4.6.

This values present a general error of 24% which will be considered as not provoking significant changes in the final concentrations of the analysed compounds. Also, the losses error for each of the severity conditions, individually, never surpassed the value of 27 % which proves the triplicates reproducibility. Having this in consideration, the remaining liquors are suitable for analysis and discussion on their application.

Table 4.6: Total mass losses (%wt), considering both liquid and solid mass fractions, not only for each of the severity conditions, but also having in consideration all the losses values.

R_0	Total losses (Liq. + Sol.) (%wt)			Error (%)
2385.2	14.11	±	1.42	10
4109.3	12.06	±	0.76	6.3
5884.8	15.39	±	4.08	27
11131.5	12.42	±	0.66	5.3
16525.3	19.25	±	4.92	26
23754.2	20.49	±	3.27	16
39628.2	15.39	±	2.05	13
64763.9	18.82	±	1.23	6.9
93064.7	17.51	±	0.47	2.7
All	16.16	±	3.86	24

4.4 Liquid phase composition

Applying the protocols described in chapter 3, it was assessed the composition of the liquid stream after the LHW fractioning step. The exact values are present in both table 4.24 and in appendix B.

As it was stated in section 4.3.1, a first indicator the raw-material is being effectively pretreated is the change in the density profile with the severity factor. This parameter might give the indication on how the concentration of solubilized components is changing with the severity factor. In fig. 4.2, it was plotted the total components concentration and the density profiles with the severity factor in order to assess if both parameters present similar behavior.

As expected, both profiles show a proportional trend with each other, meaning that density is a trustable indicator on the pretreatment extension. The only exception to this statement is the experiment using holding conditions of 180℃ and 60 min. The density average value appears to stabilise at this point, however, the standard deviation might indicate that this value can continue to follow the trend and be higher than the one plotted in this graph. Nevertheless, as a preliminary indicator, in general, density is a good indicator on the total components concentration solubilized in the liquid fraction.

Regarding the main objective of this work, meaning, sugar production, it is worthy to remark that this goal was possible to achieve using autohydrolysis, specially if the aim is to produce hemicellulosic C5 sugars. This pretretament is also known to solubilize lignin which was the case in this project as well. This also came with the setback of inhibitory compounds production, which increased with the severity imposed towards the wheat straw. The total degradation products, total solubilized lignin, as well as the monomeric and total sugars concentration profiles with the severity factor are present in fig. 4.3.

The maximum of total sugar production was reached at 180 ℃ and 60 min (12.5 ± 0.2 g/L), which intercepts with the highest C5 total sugar concentration (10.1 ± 0.2 g/L). For monomeric sugars, this

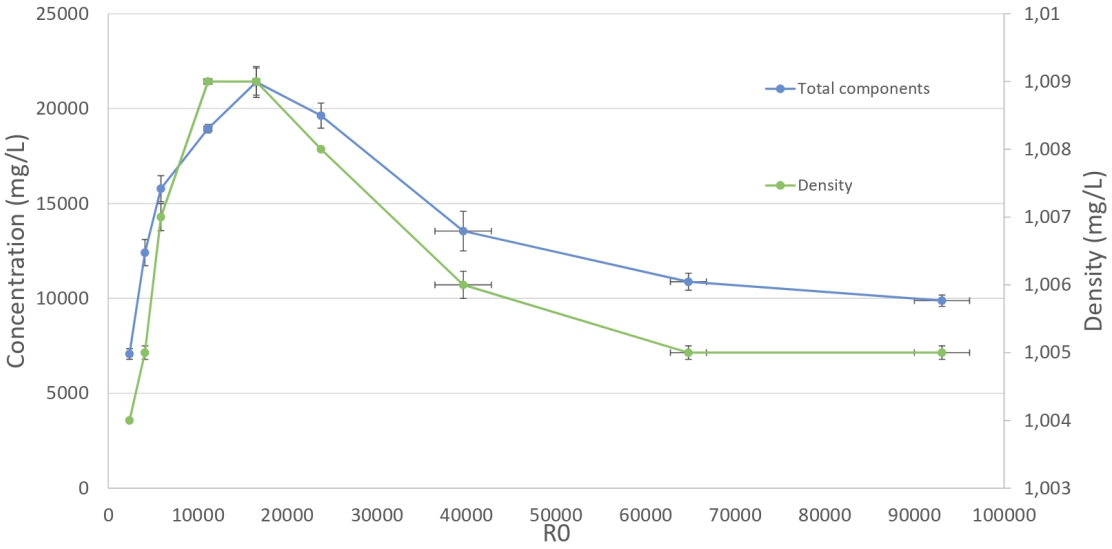

Figure 4.2: Total components concentration and density profiles with the severity factor. All the points have their respective standard deviation bars.

maximum was obtained at 180 ℃ and 90 min (3.4 $\pm$ 0.05 g/L) and it was the same condition regarding the maximum of C5 monomeric sugar concentration (3.0 $\pm$ 0.04 g/L), explained by an increase in the fractioning of the raw-material and sugar chain depolymerization with the raise in the severity factor. The rise in pentoses concentration indicates that the main structural sugar source is being the hemicellulose matrix, as expected when using this type of pretreatment. The total sugar C6 sugar concentration has its maximum at 160 ℃ and 90 min (2.45 $\pm$ 0.15 g/L), however, it has almost the same concentration value at 180 ℃ and 90 min (2.44 $\pm$ 0.15 g/L). Increasing the method severity might lead to a shift in this sugar source towards cellulose, which is evident when the C6 concentration for monomeric sugars reaches its maximum at 200 ºC and 30 min of holding parameters (0.5 $\pm$ 0.04 g/L). At this severity point, the total degradation products concentration surpasses the total sugar concentration profiles, meaning that this method purpose is not anymore sugar production, but mainly degradation products formation. When this concentration maximum occurs, the C5 concentration is already decreasing and the correspondent by-product from this type of sugars, furfural, reaches is maximum (considering the concentration mean value of the triplicates). The minimum sugar concentration, for both monomeric and total sugars assessments, coincides with the most extreme conditions for the raw material (200 ℃ and 90 min), which also

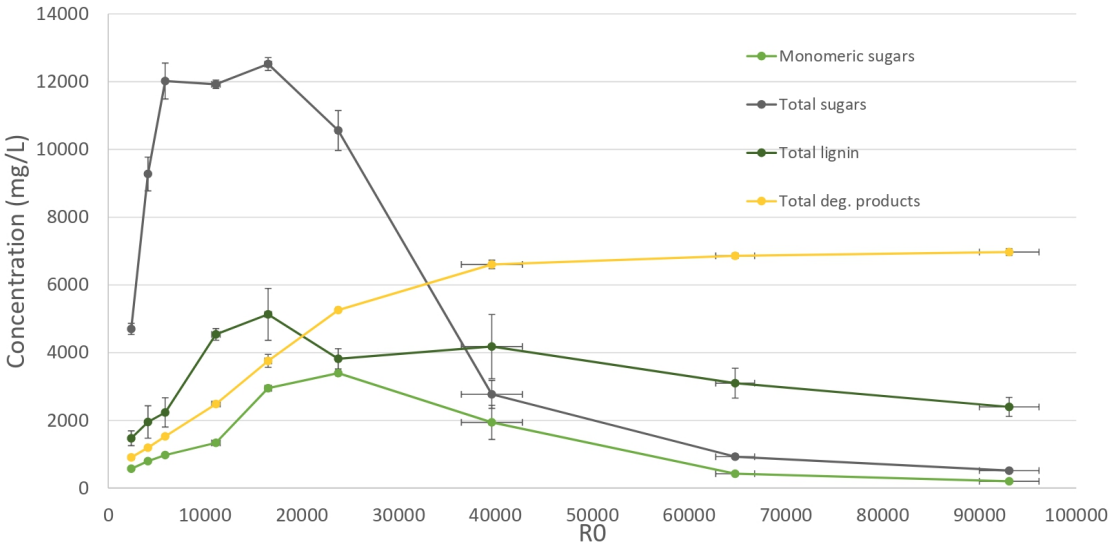

Figure 4.3: Profile of total degradation products, total solubilized lignin, monomeric and total sugars concentration with the severity factor. All the points have their respective standard deviation bars.

gives the highest concentration of HMF and acetic acid within the remaining liquid (in the case of furfural, considering the standard deviation, easily visualised in fig. 4.10. Furfural concentration also stabilises at the plateau value of around 3.2 g/L when the operational condition's aggressiveness tends to the highest studied value). This is highly expected since the degradation products use, as substrate, different subunits of the structural sugars, meaning, if less substrate is available for degradation, the degradation products formation will then stabilize. Analogously the lowest concentration for all degradation products corresponded to the run that used lesser aggressive conditions towards the raw-material, meaning, the 160 ℃ and 30 min experimental run.

The maximum of extraction of total lignin was reached at 180 ℃ during 60 min (5.1 $\pm$ 0.8 g/L), which is the also the maximum condition for total sugars production. Since the goal is not only to produce sugars, but also to maintain lignin is the solid fraction, using this condition in further valorization steps might reveal unfeasible, since lignin is being lost in the liquid stream and not being used in valorization processes of the solid stream. Analysing a less severe condition of 160℃ and 90 min of holding parameters, it only solubilizes 44% of the maximum lignin value, while it extracts 96 % of the maximum total sugar concentration. This operational condition might achieve as well the sugar requirements while it

deconstructs a considerably less amount of lignin.

This component appears to stabilise its concentration between 2.2 g/L and 5.1 g/L values even when a more harsh set of conditions is applied. Lignin concentration was always above monomeric sugar concentration, meaning that this fractioning method is also effective on removing a considerable amount of lignin from the solid wheat straw fraction. When analysing the total sugar concentration, for the more moderate conditions that were studied, this profile is always above the solubilized lignin, however, both profiles intersect when the pretreatment agressivness rises, meaning, at the 200 ℃ and 30 min operational conditions. So after this point, similarly to the degradation products concentration profile, since the total sugar concentration is always lower that lignin, it is safe to state that this set of conditions was aiming, not for sugar production, but mainly lignin solubilization. In the next subsections it is shown how this concentration data was evaluated regarding this works criterium of performance.

4.4.1 Monomeric, oligo-, and total sugars profiles analysis

Analysing both C5 and C6 sugar profiles leeds to some discussion about how the oligo- and monomeric sugars from the liquors, as the total sugar concentration, can be useful to assess how the concentration profile changes with the increase in severity for the studied conditions.

4.4.1.1 Monomeric sugars

The monomeric sugars concentration was evaluated in order to assess if this sugar enriched medium can be used in monomeric specific fermentation processes without the need of further depolymerization or detoxification steps, having in consideration the type microorganism that will use this sugar solution as substrate, for example, bacteria. The concentration profile with the severity factor for monomeric pentoses (C5), hexoses (C6), and the sum of both, are plotted in fig. 4.4.

When comparing the maximum C5 monomeric sugar concentrations in this work (3.0 $\pm$ 0.04 g/L) to the ones reported by Beisl et al. (2019) [77] (0.2 g/L), directly from the autohydrolysis liquid fraction and prior to the concentration step, it is interesting to observe that increasing the holding temperature from 120 to 180 ℃ and decreasing the holding time from 120 to 90 min results in a 15-fold increase in C5 monomeric sugar concentration values. When looking at maximum C6 monomeric sugar concentrations, Beisl et al. reports a 2.2 times higher value (1.2 g/L) when comparing to this work's concentration (0.5 $\pm$ 0.04 g/L), meaning that cellulose is being more deconstructed in Beisl et al. work than in this project. If the goal is to produce more monomeric sugars, either C5 or C6, then the protocol to follow should be the one used in this work, meaning, 180 ℃ and 90 min, with a severity factor of 23754.2 which gives a monomeric sugars concentration value of 3.4 $\pm$ 0.05 g/L. An important consideration to have, when applying this operational setup, is to combine the pretreatment with a detoxification step, if the microorganism used to ferment this sugars is susceptible to growth inhibition, namely, *P. stipitis* [93]

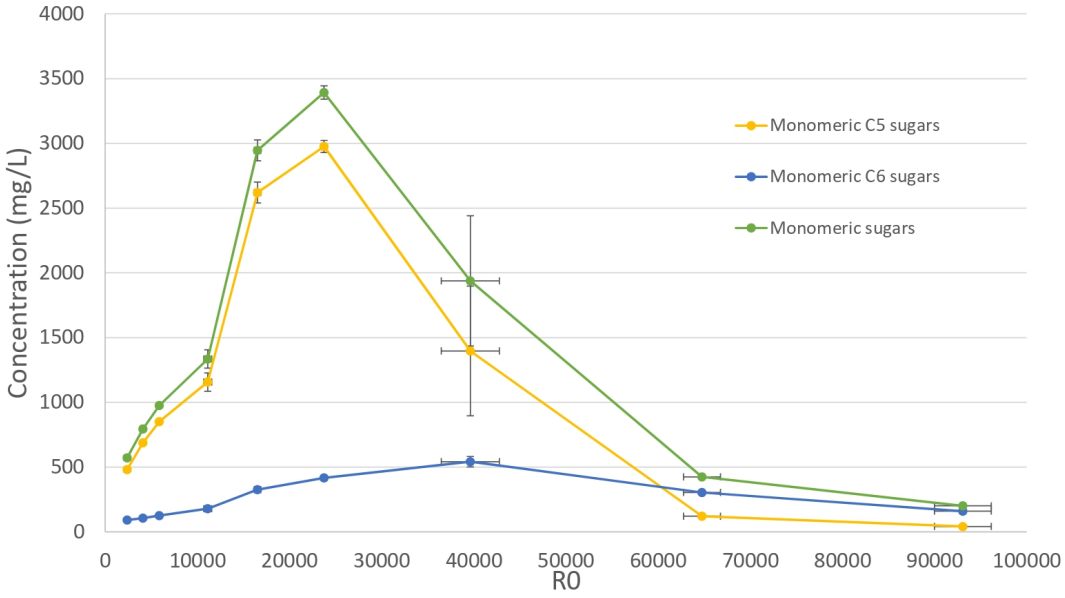

Figure 4.4: Profile of monomeric pentoses (C5), hexoses (C6) and the sum of both concentrations with the severity factor. All the experimental markers have their respective standard deviation bars.

and *S. acidocaldarius* [77] as exploited in section 4.4.2. When analysing the concentration profile of monomeric sugars originated by the pretreatment step, it is clear that the mono C5 profile is similar to the total monomeric concentration for the conditions with a severity factor less than 64763.9 (200 ℃ and 60 min of holding conditions), which is expected since the main objective of this method is to produce hemicellulosic sugars, being them, mostly, C5-type sugars. When this severity factor value is surpassed, the C6 sugar concentration exceeds the one of C5, meaning that the pretreatment method is being so harsh on the raw-material that, by this point, not only the main sugar source not anymore hemicellulose, but instead, cellulose, but also that hemicellulose-derived sugars are already being degraded into HMF and furfural. Both this factors decrease the value-added product concentration in the solid stream, reducing the overall process revenue, and, make unusable the use of this sugar solutions to fermentation processes, respectively.

Considering that the structural C5 and C6 sugar content in the raw-material was 22.4 $\pm$ 0.5 wt% and 34.2 $\pm$ 1.9 wt%, respectively, it was calculated the yield of production using eq. (3.8) for each imposed severity conditions. This calculations are present in table 4.7.

The highest yield percentage was obtained at 180℃ and 90 min (R_0 of 23754.2) for monomeric C5

Table 4.7: Monomeric pentoses (Mono C5), monomeric hexoses (Mono C6) and monomeric sugars (Mono sugars) yields (%wt) for each of the experimental conditions and the respective statistical error.

R_0	Mono C5 (%wt)	Error (%)	Mono C6 (%wt)	Error (%)	Mono Sugars (%wt)	Error (%)
2385.2	2.09 ± 0.097	4.7	0.27 ± 0.022	8.4	2.36 ± 0.100	4.2
4109.3	2.99 ± 0.078	2.6	0.31 ± 0.017	5.5	3.30 ± 0.080	2.4
5884.8	3.70 ± 0.101	2.7	0.36 ± 0.022	5.9	4.06 ± 0.104	2.6
11131.5	5.01 ± 0.330	6.6	0.52 ± 0.029	5.7	5.53 ± 0.331	6.0
16525.3	11.4 ± 0.443	3.9	0.95 ± 0.053	5.6	12.3 ± 0.446	3.6
23754.2	12.9 ± 0.374	2.9	1.21 ± 0.087	7.2	14.1 ± 0.384	2.7
39628.2	6.07 ± 2.182	35.9	1.58 ± 0.146	9.3	7.65 ± 2.187	29
64763.9	0.52 ± 0.057	10.9	0.89 ± 0.057	6.4	1.41 ± 0.080	5.7
93064.7	0.18 ± 0.022	12.4	0.47 ± 0.033	7.0	0.64 ± 0.039	6.1

sugars and 200˚C and 30 min (R_0 of 39628.2) for monomeric C6 sugars, in conformity with the highest sugar concentration for both cases. The minimum C5 monomeric sugars was observed at the most severe condition, not because the fractioning method was not able to solubilize this type of sugars, but because this components are already being converted into degradation products of the pretreatment. For the maximum yield, both of this conditions are already, not only converting a significant amount of lignin, but also surpassing the degradation products threshold imposed before. For the case of low inhibitory products concentration and low liginin conversion set of conditions, which means that at 160℃ and 90 min (R_0 of 5884.8), the amount of structural pentoses present in the solid stream is still a robust 96 % of the initial amount, meaning that there is still a lot of sugars to depolymerize into their most hydrolized form. The minimum C6 monomeric sugars was reported at the less severe condition, meaning that the fractioning method was not able to solubilize this type of sugars with such low holding conditions. Similarly to C5 monomeric sugars, after the maximum concentration is achieved, this components are being converted into degradation products as well.

Summaryzing, only 13% and 2% of the C5 and C6 initial fraction, respectively, were hydrolized, which means that if the purpose of the biorefinery was to produce monomeric sugars, a single step LHW as pretreatment still does not achieve the possible total monomeric sugar production. This total sugar concentration, either C5 or C6, will be assessed in the following section 4.4.1.2.

Microorganisms that use pentoses in their metabolic pathways usually require also a smaller hexose fraction in order to start up fermentations. This can be quantified by the ratios between monomeric pentoses and monomeric hexoses (C5/C6), monomeric pentoses and monomeric sugars (C5/Mono sugars) and monomeric hexoses and monomeric sugars (C6/Mono). All this values, for each experimental condition, are presented in table table 4.8.

Computing the ratios for the sugar concentrations reported by Beisl et al. [77], giving the values of 0.17, 0.14 and 0.86 for the C5/C6, C5/Mono and C6/Mono, respectively, and comparing those ratios to the ones described in table 4.8, it is observed that they are only comparable for high severity factor

Table 4.8: Ratio betwenn monomeric pentoses and monomeric hexoses (C5/C6), monomeric pentoses and monomeric sugars (C5/Mono sugars) and monomeric hexoses and monomeric sugars (C6/Mono) for each of the experimental conditions and the respective statistical error.

R_0	C5/C6	Error (%)	C5/Mono	Error (%)	C6/Mono	Error (%)
2385.2	5.255 ± 0.395	7.5	0.840 ± 0.044	5.3	0.160 ± 0.012	7.3
4109.3	6.465 ± 0.074	1.2	0.866 ± 0.011	1.3	0.134 ± 0.001	1.0
5884.8	6.812 ± 0.180	2.6	0.872 ± 0.015	1.7	0.128 ± 0.003	2.6
11131.5	6.488 ± 0.410	6.3	0.866 ± 0.070	8.1	0.134 ± 0.007	5.5
16525.3	8.049 ± 0.266	3.3	0.889 ± 0.037	4.1	0.111 ± 0.003	3.0
23754.2	7.146 ± 0.353	4.9	0.877 ± 0.019	2.2	0.123 ± 0.006	4.9
39628.2	2.581 ± 0.946	37	0.721 ± 0.319	44	0.279 ± 0.075	27
64763.9	0.396 ± 0.044	11	0.284 ± 0.032	11	0.716 ± 0.037	5.1
93064.7	0.257 ± 0.033	13	0.205 ± 0.026	13	0.795 ± 0.049	6.1

values, which means, higher than 64764.9. This range of severity factor provides a sugar solution with a toxicity higher than the threshold for degradation products. An approach to make this values converge is supplementing the sugar solution with other sugar types so the sugar ratio requirements could be achieved and making this solution a feasible substrate for fermentation processes. Nevertheless, an incubation study, prior to industrial scale is always obligatory in order to assess cellular growth with this sugar solution. This assessment should provide the data needed to decide which microorganism and sugar solution suits better for the biomass refinery implementation.

In order to modulate the monomeric sugars profile with the severity factor, using eq. (3.5), it was assessed how the experimental data fitted the polynomial models. The parameters for modeling monomeric sugars are described in table 4.9, as well as the respective correlation coefficient (R^2 and the severity factor domain of application.

Table 4.9: Monomeric (Mono) sugars modeling parameters, for pentoses (C5), hexoses (c6) and the sum of both, as well as the respective correlation coefficient (R^2) and the severity factor domain of application.

	a_5	a_4	a_3	a_2	a_1	a_0	R^2	Domain
Mono C5	-	-	-8.00E-10	3.00E-05	-0.1893	910.51	0.9708	[2385.2;23754.2[
	-	-	-	1.00E-06	-0.1578	6140.7	0.9992	[23754.2;93064.7]
Mono C6	-2.00E-21	6.00E-16	-5.00E-11	2.00E-06	-4.20E-03	9.41E+01	0.9938	[2385.2;93064.7]
Mono Sugars	-	-	-8.00E-10	3.00E-05	-0.2047	1033	0.9738	[2385.2;23754.2[
	-	-	-	9.00E-07	-0.1526	6522.6	0.9996	[23754.2;93064.7]

The correlation coefficient (R^2) is always higher than 0.97 which indicates a good fitting between the experimental values and the predicted model concentration, in the respective domain. Computing the eq. (3.5) with the parameters described in table 4.9, it is given the predicted value of monomeric sugar concentrations, depending on the severity factor and its respective domain of application. In the cases of monomeric C5 sugars and total monomeric sugars, it was considered a 2-phase polynomial model. The concentration values, as the respective relative percent deviation, are described in table 4.10.

Analysing the deviation values, it is observed an increase when using severity factor values close

Table 4.10: Concentration modelling of monomeric (Mono) sugars, for pentoses (C5), hexoses (C6) and the sum of both, as well as the respective relative percent deviation (RPD).

R_0	Mono C5 (mg/L)		RPD		Mono C6 (mg/L)	RPD	Mono Sugars (mg/L)		RPD	
2385.2	618.8	-	25	-	94.8	3.7	704.6	-	21	-
4109.3	583.7	-	16	-	107.3	0.9	642.9	-	21	-
5884.8	672.4	-	23	-	129.2	3.3	704.3	-	32	-
11131.5	1417.2	-	20	-	235.1	27	1368.3	-	2.5	-
16525.3	2364.6	-	10	-	387.5	17	2232.6	-	28	-
23754.2	2618.8	2956.5	13	0.7	628.6	41	2375.5	3405.5	35	0.4
39628.2	-	1457.8	-	4.3	1241.1	79	-	1888.7	-	2.6
64763.9	-	115.3	-	4.3	2905.5	162	-	414.6	-	2.3
93064.7	-	116.1	-	96	7769.4	192	-	115.9	-	54

to the domain limiting values, reaching a maximum 192 % of deviation. In order to decrease it, a more extended assessment should be made, which means, fixing more experimental conditions in order to get more data points for further modulation.

4.4.1.2 Total sugars

In this section, the total sugars concentration was evaluated in order to assess if the pretreated liquors can be used in cellular growth and metabolic fermentation process that are not monomeric specific. Since the previous study only shows the extracted sugars that are in a monomeric form, this total sugars study allows to assess how much of the total sugar molecules are being, effectively, removed from the LCB structure. The total pentoses (C5), total hexoses (C6) and the sum of both concentrations profile with the severity factor are plotted in fig. 4.5

In this case, analysing the concentration profile of the total sugars, meaning, all the sugars in the less polymerised (more hydrolyzed) condition possible, it is clear that the total C5 profile is similar to the total sugar concentration for the conditions with a severity factor less than 39628.2 (200 ℃ and 30 min of holding conditions), which is again and indicator that LHW method is producing, mainly C5 sugars from hemicellulose. This concentration value is exceeded by the C6 sugars by the same reasons described in section 4.4.1.1, nonetheless, it happened now at a lower aggressive condition (200ºC for 30 min) than before. This can be explained by changes in the degree of polymerization comparing both type of sugars. For the first case, there was a higher concentration of large polymer molecules (e.g.: oligosacharides) susceptible to be hydrolyzed and the energy supplied to the system, via heat exchange, was being used to depolymerize this molecules into monomers. Since in the total sugar assessment all the samples have been hydrolyzed *a priori* (see section 3.3.7) the only incidence of the thermal energy is into the hydrolyzed sugars (monomers) which will led to the conversion into pretreatment degradation products at a higher rate than before.

Comparing the maximum total C5 sugar concentrations in this work (10.1 $\pm$ 0.2 g/L) to the ones

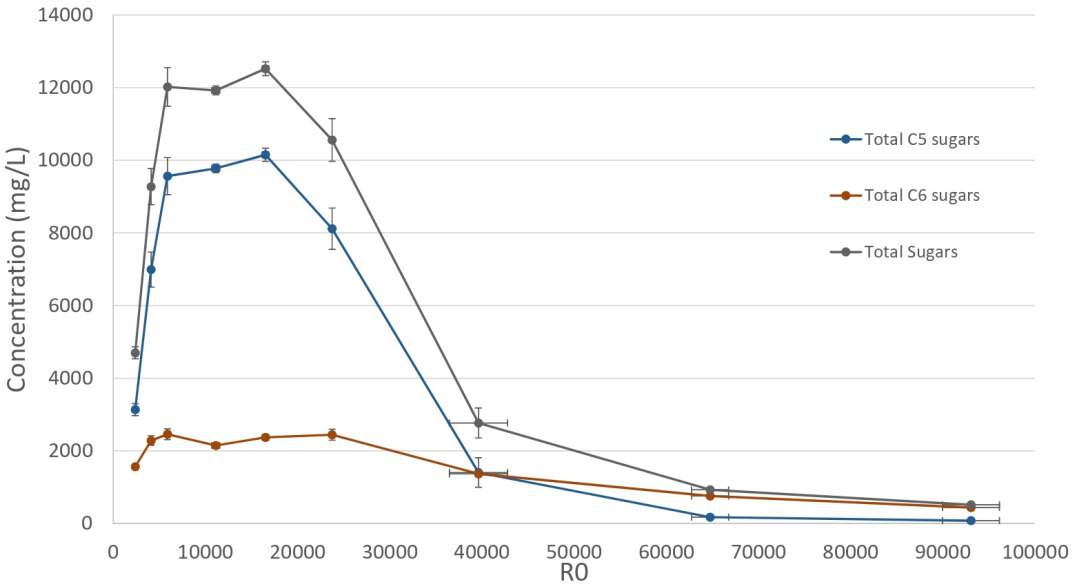

Figure 4.5: Profile of total pentoses (C5), total hexoses (C6) and the sum of both concentrations, with the severity factor. All the experimental markers have their respective standard deviation bars.

reported by Beisl et al. (2019) [77] (0.6 g/L), similarly to section 4.4.1.1, it is again observed that increasing the holding temperature from 120 to 180℃ and decreasing the holding time from 120 to 60 min results in a even higher 17-fold increase in total C5 sugar concentration values. For total C6 sugar concentrations, Beisl et al. now reports less concentration of this type of sugars (2.1 g/L) when comparing to this work's concentration (2.5 $\pm$ 0.15 g/L). An interesting assessment to make is that increasing the holding temperature from 120 to 160℃ and decreasing the holding time from 120 to 90 min results only in a 20% increase in total hexose sugar concentrations. If the goal is to produce more total sugars, either C5 or C6, then the protocol to follow should be the one used in this work, meaning, 180 ℃ and 60 min, with a severity factor of 16525.3 which gives a total sugars concentration value of 12.5 $\pm$ 0.2 g/L. Again, this condition is already above the thresholds reported previously by Nigam et al. (2001) for *P. stipitis* [93] and Beisl et al. (2019) for *S. acidocaldarius* [77].

The maximum C5 total sugar concentration (10.1 $\pm$ 0.2 g/L) was obtained at a severity factor of 16525.4 (180℃ for 60 min), however, it has a similar concentration value to other conditions with a lower severity factor, more specifically, using the set temperature at 180ºC and 60 min holding time (R_0 of 11131.5), and 160 ºC for 90 min (R_0 of 5884.8). This indicates that, at 160 ℃ of temperature and

90 min holding time, the hemicellulose sugars possible to be solubilized into the liquid fraction reaches a plateau of maximum extraction. Since the rate of production is decreasing, despite of having the extraction maximum only at 180 ℃ and 60 min holding time, it indicates an increase in secondary reactions, forming degradation products from this hemicellulosic sugars. After the concentration maximum is reached, the pretreatment method is being so aggressive on the raw-material that, by this point, the rate of sugar production from hemicellulose hydrolysis is surpassed by the rate of degradation products formation. This can be confirmed by looking at the previous fig. 4.3, when comparing the total degradation products concentration with the total sugars produced. This degradation products surpass the total sugar concentration for the condition of 200℃ for 30 min (R_0 of 39628.2) which is a clear evidence of the shift in the reaction equilibrium towards degradation products formation.

When looking at the C6 sugar concentration profile, after it reaches is maximum at the severity factor of 5884.8, there is a decrease in the total sugar concentration by 13% when comparing to the next harsh operation condition (R_0 of 11131.5), however, the sugar concentration increases again, in 10 %, for the following condition, with the severity factor value of 16525.4. This could be an indicator of cellulose disruption and subsequent increase in the production of C6 sugars, mainly glucose, having this matrix has sugar source. The HMF formation, that has its origin from C6 sugars, needs a higher severity factor value to be produced than the case of furfural, which use C5 sugars as substrate. This can be justified since, in this conditions, the energy transferred into the reactional mixture is still being used for the disruption of cellulose and not for the production of degradation products. To confirm this hypotheses, the glucose concentration profile is studied separately in section 4.4.1.4. After the condition that gives the maximum of total sugar concentration, the more harsh it was towards the wheat straw, the more this concentration decreases, meaning that the pretreatment method is being so aggressive on the raw-material that, by this point, the applied protocol is not producing sugars, but instead, degradation products.

Considering, again, the structural C5 and C6 sugar content in the raw-material, presented in section 4.4.1.1, it was calculated the yield of production using eq. (3.8) for each imposed severity conditions, now for the total solubilized sugars. This calculations are present in table 4.11.

The highest yield percentage was obtained at 180℃ and 60 min (R_0 of 16525.3) for total C5 sugars and 160˚C and 90 min (R_0 of 5884.8) for total C6 sugars, in conformity with the highest sugar concentration for both cases. The minimum for both total C5 and C6 sugars was observed at the most severe condition, not because the fractioning method was not able to solubilize this type of sugars, but because this components are already being converted into degradation products of the pretreatment. For the maximum yield of total C5 production, this working conditions are already, not only converting a significant amount of lignin, but also surpassing the degradation products threshold imposed before, despite a production yield of around 44% of the total C5 sugars available in the raw-material. Analysing the case

Table 4.11: Total pentoses (Total C5), Total hexoses (Total C6) and Total sugars yields (%wt) for each of the experimental conditions and the respective statistical error.

R_0	Total C5 (%wt)	Error (%)	Total C6 (%wt)	Error (%)	Total Sugars (%wt)	Error (%)
2385.2	13.65 ± 0.783	5.7	4.57 ± 0.257	5.6	18.22 ± 0.824	4.5
4109.3	30.40 ± 2.219	7.3	6.66 ± 0.530	8.0	37.06 ± 2.281	6.2
5884.8	41.53 ± 2.439	5.9	7.16 ± 0.581	8.1	48.69 ± 2.507	5.1
11131.5	42.37 ± 1.149	2.7	6.24 ± 0.353	5.7	48.61 ± 1.202	2.5
16525.3	43.97 ± 1.319	3.0	6.89 ± 0.408	5.9	50.86 ± 1.381	2.7
23754.2	35.21 ± 2.611	7.4	7.11 ± 0.586	8.2	42.32 ± 2.676	6.3
39628.2	6.10 ± 1.767	29	3.97 ± 0.307	7.7	10.07 ± 1.794	18
64763.9	0.75 ± 0.048	6.4	2.20 ± 0.140	6.4	2.95 ± 0.148	5.0
93064.7	0.33 ± 0.014	4.3	1.28 ± 0.093	7.3	1.61 ± 0.095	5.9

that gives low inhibitory products concentration and low liginin conversion, meaining, 160 ℃ and 90 min, the amount of solubilized pentoses is reduced, but only to close to 42 % of the initial amount, which means that decreasing in only 2% C5 total sugar production, can turn the method viable for fermentable sugars prodution even when considering the imposed restrains regarding degradation products thresholds. It is also interesting to register that the highest total C6 sugar yield is obtained at this condition (R_0 of 5884.8) which can be an application advantage. Adding to this, this condition not only requires a lower energy supply than the one that gives the highest sugar concentration, but also, working using this conditions allows the cellulose matrix to be maintained as immaculate as possible and within the solid stream, for future valorization. This yield value also proves that LHW is not effective in extracting C6 sugars since around 93% of this sugars are still in the solid stream and raising the severity in the conditions of the method does not produce more sugars than in the moderate ones. This sugar production also proves that LHW is specific it therms of the sugar types that are being extracted from the raw-material, meaning, this pretreatment is useful to solubilize sugars from an hemicellulosic source. The purpose of the biorefinery on the sugar production in their solubilized form is achieved. Nevertheless, the application as source of fermentable sugars should be taken into consideration since not all the microorganisms are capable of metabolize oligosugars.

Similarly to the previous section, the ratios of total pentoses and total hexoses (C5/C6), total pentoses and total sugars (C5/Total) and total hexoses and total sugars (C6/Total) are presented in table table 4.8. Computing the ratios for the sugar concentrations reported by Beisl et al. [77], giving the values of 0.28, 0.22 and 0.78 for the C5/C6, C5/Mono and C6/Mono, respectively, and comparing those ratios to the ones described in table 4.12, it is observed that they are only comparable for high severity factor values, which means, higher than 64764.9. Similar to the monomeric sugar ratios, this range of severity factor provides a sugar solution with a toxicity higher than the threshold for degradation products. The approaches described previously, to make this values converge, and so, achieving the sugar ratio requirements, are applied in this case as well.

Table 4.12: Ratio betwenn total pentoses and total hexoses (C5/C6), total pentoses and total sugars (C5/Total) and total hexoses and total sugars (C6/Total) for each of the experimental conditions and the respective statistical error.

R_0	C5/C6	Error (%)	C5/Total	Error (%)	C6/Total	Error (%)
2385.2	2.003 ± 0.107	5.4	0.667 ± 0.042	6.3	0.333 ± 0.012	3.7
4109.3	3.061 ± 0.275	9.0	0.754 ± 0.066	8.7	0.246 ± 0.019	7.9
5884.8	3.892 ± 0.312	8.0	0.796 ± 0.055	6.9	0.204 ± 0.015	7.4
11131.5	4.551 ± 0.085	1.9	0.820 ± 0.013	1.6	0.180 ± 0.003	1.8
16525.3	4.281 ± 0.124	2.9	0.811 ± 0.019	2.3	0.189 ± 0.005	2.7
23754.2	3.322 ± 0.310	9.3	0.769 ± 0.069	9.0	0.231 ± 0.019	8.3
39628.2	1.029 ± 0.303	29	0.507 ± 0.165	33	0.493 ± 0.078	16
64763.9	0.228 ± 0.015	6.8	0.186 ± 0.012	6.6	0.814 ± 0.035	4.3
93064.7	0.176 ± 0.011	6.0	0.150 ± 0.008	5.5	0.850 ± 0.054	6.4

In order to modulate the total sugars profile with the severity factor, using eq. (3.5), it was assessed how the experimental data fitted the polynomial models. The parameters for modeling total sugars are described in table 4.13 as well as the respective correlation coefficient (R^2 and the severity factor domain of application.

Table 4.13: Total sugars modeling parameters, for pentoses (C5), hexoses (c6) and the sum of both, as well as the respective correlation coefficient (R^2) and the severity factor domain of application.

	a_5	a_4	a_3	a_2	a_1	a_0	R^2	Domain
Total C5	1.29E-19	-3.39E-14	3.28E-09	-1.39E-04	2.2041	-475.63	0.9709	[2385.2;93064.7]
Total C6	-	-5E-16	1E-10	-0.000007	0.1506	1528.7	0.9252	[2385.2;93064.7]
Total Sugars	1.26E-19	-3.38E-14	3.34E-09	-1.45E-04	2.3398	1092.8	0.965	[2385.2;93064.7]

The correlation coefficient (R^2) is always higher than 0.925 which indicates a good fitting between the experimental values and the predicted model concentration, in all of the domain of experimentation. Computing the eq. (3.5) with the parameters described in table 4.13, it is given the predicted value of total sugar concentrations, depending on the severity factor. The concentration values, as the respective relative percent deviation, are described in table 4.14.

Table 4.14: Concentration modelling of total sugars, for pentoses (C5), hexoses (C6) and the sum of both, as well as the respective relative percent deviation (RPD). Negative values were marked with a (-) symbol.

R_0	Total C5 (mg/L)	RPD	Total C6 (mg/L)	RPD	Total Sugars (mg/L)	RPD
2385.2	4034.3	25.1	1849.4	16.7	5893.1	22.6
4109.3	6452.5	8.0	2036.1	11.4	8481.5	8.9
5884.8	8310.0	14.0	2192.3	11.4	10481.6	13.7
11131.5	10861.5	10.5	2468.0	13.9	13280.8	10.8
16525.3	10421.8	2.6	2519.8	6.1	12868.7	2.7
23754.2	7594.5	6.6	2337.4	4.4	9814.2	7.3
39628.2	1709.6	19.7	1494.1	9.2	2920.6	5.4
64763.9	831.0	131.4	289.6	89.1	661.5	33.4
93064.7	2168.6	186.3	n/d	n/d	n/d	n/d

Analysing the deviation values, it is observed an increase when using severity factor values close to the domain limiting values, reaching a maximum of around 186 % of deviation. Negative concentration values were also predicted using this models, for hexoses and total sugar concentrations. In order to decrease the RPD value and avoid getting negative values, a more extended assessment should be made, which means, fixing more experimental conditions in order to get more data points for further modulation.

4.4.1.3 Oligomeric sugars

The oligosugars, correspondent to the difference between total and monomeric sugars concentrations, gives us, not only the perspective and application of this liquid stream in non-monomeric fermentations processes or food industries, but also, how much sugar molecules are being removed from the LCB structure in the form of oligomers.

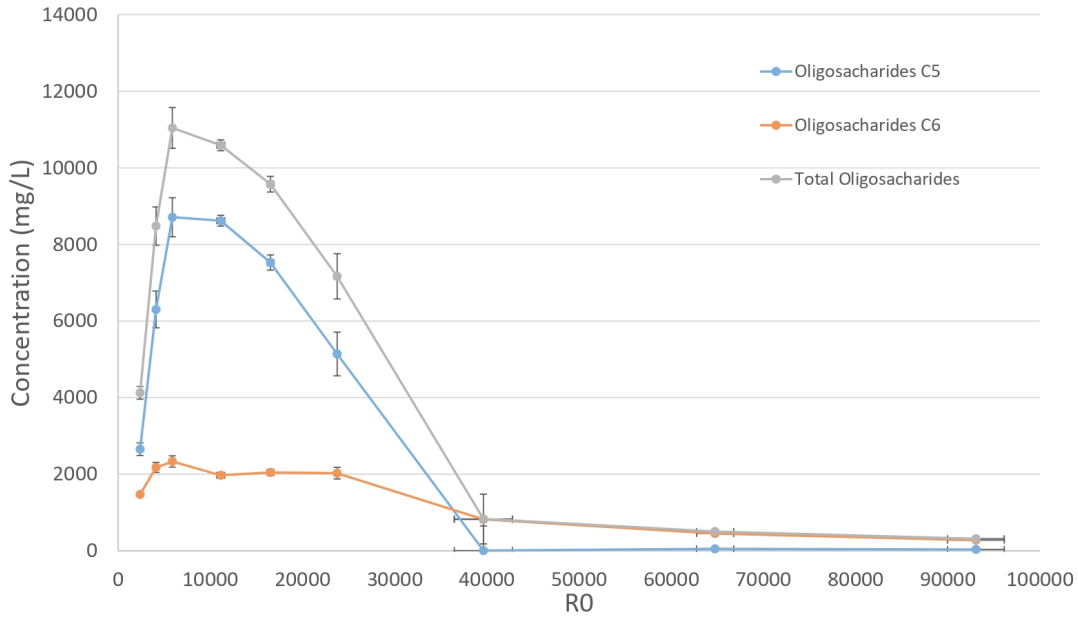

Figure 4.6: Profile of oligomeric pentoses (C5), oligomeric hexoses (C6) and the sum of both concentrations, with the severity factor. All the experimental markers have their respective standard deviation bars.

Analyzing the total and monomeric sugar plots it is clear that both curves converge at a certain value of severity factor (39628.2 for the holding conditions of 200 ℃ and 30 min) and, tend to decrease

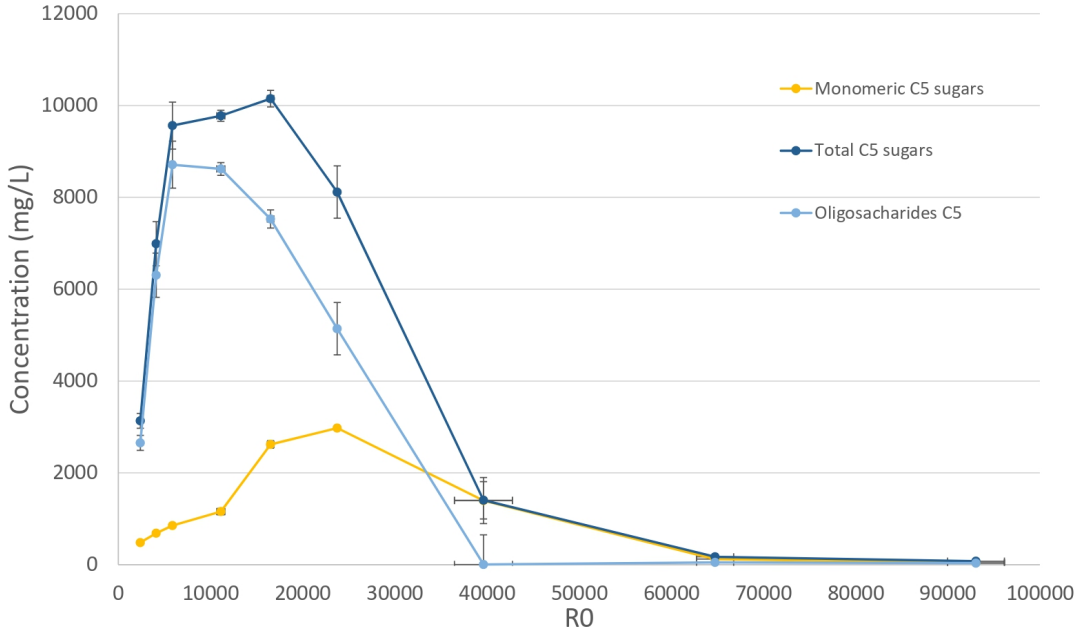

Figure 4.7: Profile of monomeric pentoses (C5), oligopentoses (C5), and the sum of both concentrations, with the severity factor. All the experimental markers have their respective standard deviation bars.

when the temperature and time keep increasing after the mentioned severity factor. This can only mean that at 200 ℃ all the oligomeric sugars have been hydrolyzed into monomeric sugars. The oligomeric C5 sugar plot well justifies this statement, since the sugar concentration intercepts with the horizontal axis, when considering this type of polymeric sugars, for this set of conditions. In the case of the C6 oligomeric sugars, it is presented a similar behavior between analogous curves, however, in this case, the monomeric and total sugar plots never get to intercept for the conditions here analysed. This is also another indicator that, after a certain severity factor value, the energy that the lignocellulosic material is being exposed to is being used for, not only the production of inhibitory products, but also the disintegration of the cellulose matrix, and consequent production of C6 sugars that compensate the loss of C6 oligosugars and delay their complete degradation. This explains the increase in the monosugars concentration to both C5 and C6 monomeric sugars when the oligosugars concentration starts to decrease. Out of this range of severity condition, it is speculation make a definite statement about the concentration profile behavior when the manipulated variable here in place, meaning when the R_0, tends to infinity, nonetheless, it is expected to also converge to the null value, similarly to the previous case.

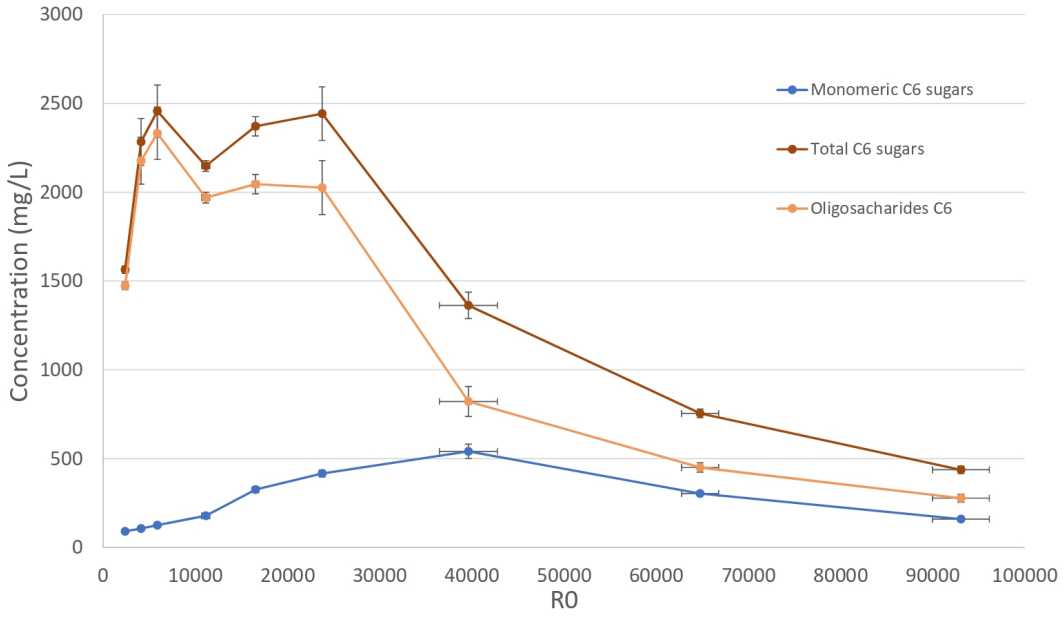

Figure 4.8: Profile of monomeric hexoses (C6), oligohexoses (C6), and the sum of both concentrations with the severity factor. All the experimental markers have their respective standard deviation bars.

It is interesting to analyse also some operational conditions that may be indicators of what is happening to the raw-material in terms of the extracted sugar source. Firstly, the maximum oligosugars concentration value, that correspond to the holding conditions 160 ℃ and 90 min for both C5 and C5, not only is a low energy demanding condition, but also, low degradation products concentrations are achieved using the respective severity factor (5884.8). When looking at the following condition, 180 ℃ and 30 min, it implicates an 2-fold increase in the severity factor, surpasses both thresholds imposed by Delgenes et al. (1996) [94] and Nigam et al. (2001) [93] and the outcome is having the same oligosugars concentration (considering the standard deviation) and an increase in 36 % in monomeric sugars concentration. If the biomass refinery objective is to produce oligomeric sugars with the less degradation products concentration, then the set-up conditions to apply impose themselves to be 160 ℃ of holding temperature and 90 min holding time.

The oscillating behavior in the concentration of C6 oligosugars for the severity factor range between 5884.8 and 23754.2 will be described further in section 4.4.1.4 by the study of cellulose concentration profile to assess cellulose fractioning.

4.4.1.4 Assessing cellulose fractioning by glucose concentration profiles

One of the project's objectives is maintaining cellulose structure as much as possible. A good indicator that this is being achieved is the glucose concentration profile. Monomeric glucose and Glucoligosacharides concentration profiles are plotted in fig. 4.9 and analysed in this section.

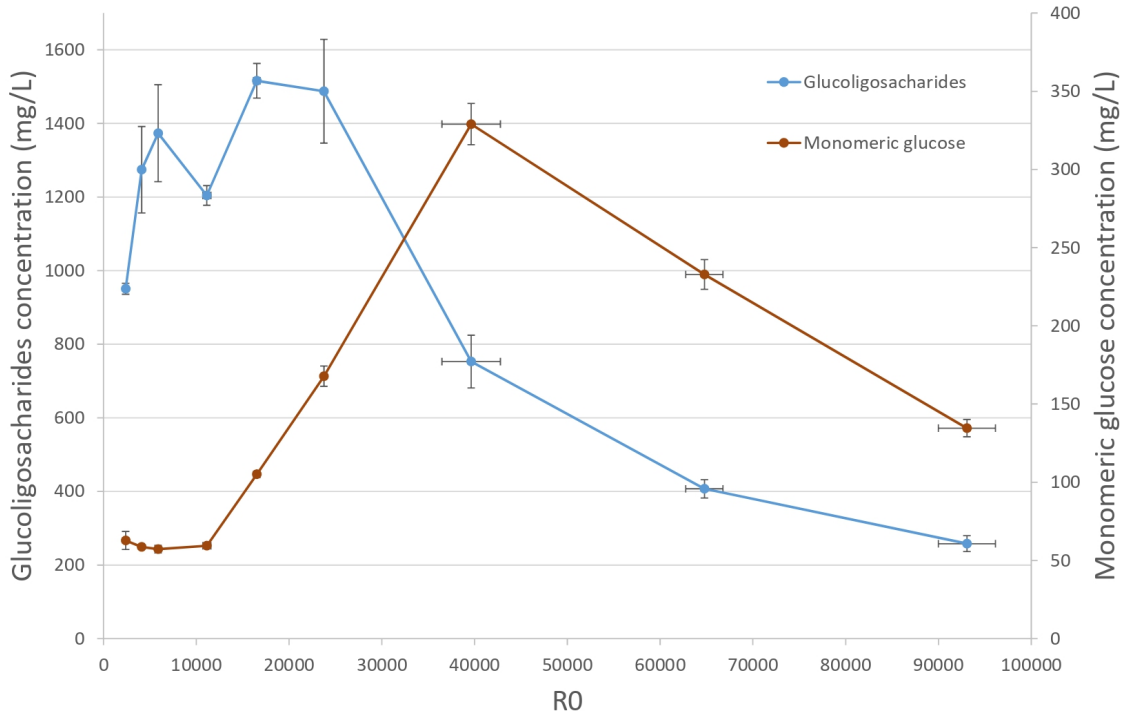

Figure 4.9: Profile of glucoligosacharides (left, or primary, axis) and monomeric glucose (right, or secondary, axis) concentration for the analyzed conditions and fixed severity factor R_0. The glucoligosacharides concentration was calculated by the subtraction of the total glucose concentration and monomeric glucose concentration values. All the points have their respective standard deviation bars.

As stated in chapter 2, despite hemicelluose being an heteropolymer that contain both hexoses and pentoses, LHW main aim is to solubilize C5 sugars. Nevertheless, C6 sugars are also identified when preforming analytics, even at the lesser severity condition, meaning that hexoses are also being produced from hemicellulose. This C6 sugar concentration, however, is far smaller than the C5 sugar concentration, as described before, meaning, that if the glucose concentration value suffers a sudden increase, with raising the severity factor, that points to the start of the fractioning of the cellulose matrix and monomerization of cellulosic glucose. Recent studies have reported a fraction of cellulosic glucose concentrations in the resulting liquid fraction, using LHW as pretreatment [95]. That is precisely what happens in this study. The monomeric glucose concentration, evaluated in the obtained sugar liquors,

shows a concentration plateau in the first 4 less harsh conditions, meaning that the monomeric glucose also present in hemicelullose (but in far lesser concentrations) is solubilized even when the smoothest condition is applied and it maintains stable until 180 ºC and 30 min (R_0 of 11131.5). After that point, the glucose concentration value suffers a sudden increase, that only stops when the operational conditions reaches 200 ºC for 30 min (R_0 of 39628.2). At those conditions, with the increase of holding time, and consequently, R_0, the glucose concentration in the extract decreases since now the reactional balance tends to the formation of HMF. This means that the overall depolymerization, specifically from cellulose at this conditions, produces now less glucose than the one being degraded into the respective inhibitory product.

Analysing the glucoligosacharides this increase on glucose polymers fractioning becomes even more evident. This concentration increases with the holding time for the runs that had a holding temperature of 160 ºC (R_0 between 2385.2 and 5884.8). After that point, for the severity factor value of 11131.5, this concentration suffers a decrease on its value. This variation can be justified by the hydrolyzation of this large sugar molecular chains into small ones, nevertheless, since the monomeric sugar concentration does not increase significantly, this depolymerization, at this conditions, does not produce an extended monomerization reaction of hemicellulosic sugars, but something in between.

Observing the following condition, with a R_0 of 16525.3, an increase in both concentrations profile is observed, well justified by the reaching of the cellulosic source of glucose, either in large or monomeric molecular forms. As expected, the glucoligosacharides concentration decreases again for the condition of 180 ˚C and 60 min (R_0 of 16525.3), even with the monomeric glucose concentration increase for this set up conditions, which indicates now the depolymerization, at the monomers extent, of the recent hydrolysed cellulosic matrix.

Both concentration profiles give a proper contribution when choosing the operational conditions to work with wheat straw. The conditions that does not interfere significantly in the raw-material's cellulose matrix are the ones with lesser severity factor than 11131.5 (180 ˚C and 30 min), however, at this point, the concentration of glucoligosacharides that are being degraded into smaller glucose polymers is significant at the point of decreasing the total concentration of this type of sugars. So, if the objective is only to have a high amount of total sugars in solution, regardingless of their form, there is no need to spend energy to depolymerize them, meaning, the more suitable operational condition is the one reaching the highest concentration of glucoligosacharides, and this takes place at the conditions of 160 ˚C and 90 min (5884.8 of R_0). This condition also allows cellulose to be maintained on its structure, avoiding, as far as possible, its fractioning.

4.4.2 Degradation products analysis

Since degradation products may condition future fermentation steps, the next criterium to consider needs to be the already cited degradation products concentration thresholds.

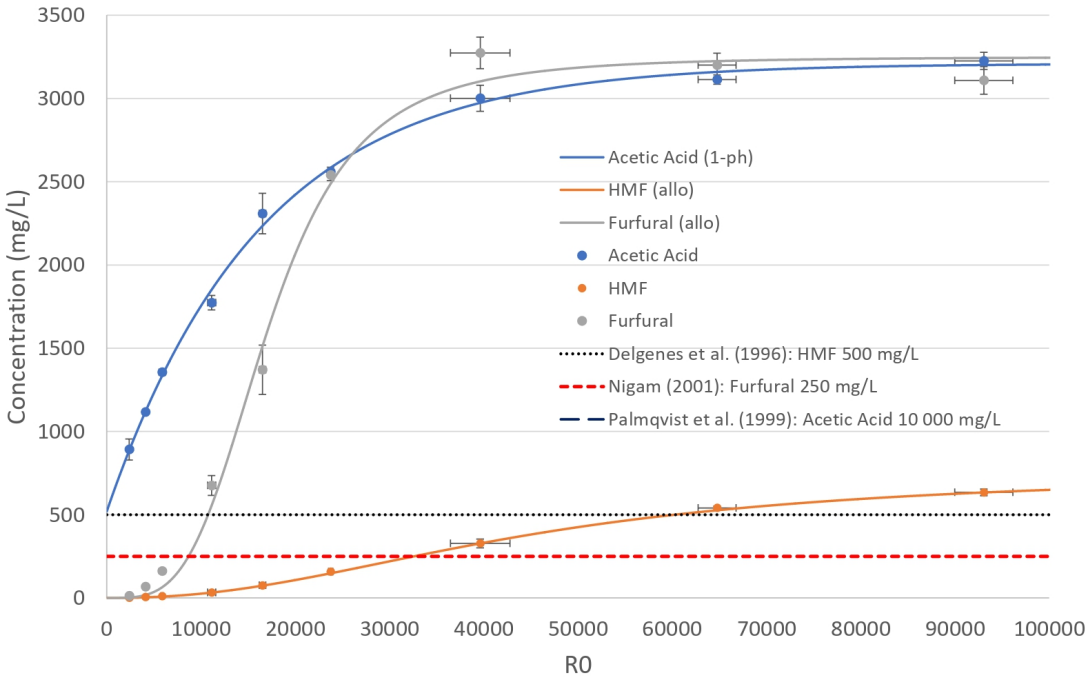

Figure 4.10: Degradation products experimental data and models with the corresponding severity factor (R_0). It is plotted the best fitting model for each by-product having in consideration the table 4.16, meaning: one-phase association model (1-ph) for acetic acid and allosteric sigmoidal model (allo) for both HMF and furfural. Inhibitory concentration thresholds are also drawn using horizontal lines. Acetic acid limit is not represented since a concentration of 10 g/L of this degradation product is out of this project scope for all the experimental conditions. All the experimental markers have their respective standard deviation bars.

Considering the previous studies of Palmqvist et al. (1999) [75], that states that acetic acid presents inhibitory behavior on yeast growth only when it reaches the concentration of 10 g/L, all the conditions are within this limit. The raise on this acid concentration can even be helpful since it auto-catalyzes the hydrolysis of hemicellulose, resulting in a more effective hemicellulosic sugar solubilization and extraction [25]. With HMF, it seems that this degradation product also did not interfere, at least significantly, in the cellular growth. Sanchez & Bautista (1988) [76] stated that HMF only had the effect of increasing the yeast culture lag phase. Other approach was made by Delgenes et al. [94], cited by Mussato et al. (2004) [32], reported that when using the yeast *Pichia stipitis*, a 43 % reduction in cellular growth is

achieved when the concentration of 0.5 g/L HMF is achieved. Nevertheless, even considering this more conservative approach for this study, this value is only achieved when using the condition of 200 ℃ and 60 min holding time (or higher). When analysing the outcome concentration for furfural it presents a higher concentration in all the operational conditions when compared to the previous component. This was expected since furfural is the decay product of C5 sugars which are the main type of sugars from the hemicellulosic matrix. On the other hand, HMF is the degradation product from C6 sugars, the main component of cellulose. For furfural, again, in Delgenes et al. (1996) work [94], still with the *Pichia stipitis*, reported a reduction of 25% on cellular growth when furfural concentration reached 0.5 g/L. For the same yeast type, Nigam et al. (2001) [93] claim that a concentration of 0.25 g/L reduces 10 % in the ethanol production yield. When analysing the concentration of furfural in this work, for both publications, this is only obtained when using the 160 ℃ as holding temperature. For the maximum holding time of this set of experiments (90 min) the furfural concentration is still within range. For another type of yeast, *Saccharomyces cerevisiae*, the work of Sanchez & Bautista (1988) [76] presents a higher threshold, stating that furfural only begins to affect cellular growth at 1.5 g/L. If this microorganism is used in further valorization processes, that could mean to use only a maximum of harshness conditions, for the pretreatment of wheat straw, of 180 ℃ and 60 min holding time.

In order to better predict the degradation products concentration with the severity factor it was evaluated how the data points fitted with the one-phase association and allosteric sigmoidal models. The model parameters for each of the models are presented in table 4.15. The best fitting models are plotted in fig. 4.10.

Table 4.15: Degradation products models parameters and the respective correlation coefficient (R^2)

	One phase association				Allosteric sigmoidal		
	Acetic Acid	HMF	Furfural		Acetic Acid	HMF	Furfural
y_0 (mg/L)	519.7	-54.63	-778.5	a (mg/L)	3759	756.5	3250
Plateau (mg/L)	3210	1322	3385	h	0.8944	2,236	3.644
K	6.139E-05	7.855E-06	5.243E-05	b	3973	2.503E+10	2.701E+15
R^2	0.9926	0.9829	0.9548	R^2	0.9859	0.9970	0.9911

When analysing the models, it is clear that the parameters definitions for $Plateau$, in the case of one-phase association and a in the allosteric sigmoidal are similar, so the concentration values (mg/L), given by this parameters are expected to be similar. In fact, applying the eq. (A.11), this parameters, only deviate from each other 16% and 4% for acetic acid and furfural, respectively. However, in the case of HMF there is a deviation of 65%. This can be explained since the HMF concentration did not yet reached a maximum (plateau) for the highest severity factor that was studied, which means that, applying the models out of the range of this operational conditions, regarding this degradation product, led to extrapolations, and consequently, discrepancies, in the values parameterization.

Specifically for one-phase association model, y_0 gives the degradation product concentration for a null severity factor, meaning, the condition of 100 ˚C and no holding time. In the case of acetic acid, this model predicts a concentration of 0.5 g/L for this condition, meaning, that the acetyl groups are already being detached from the hemicellulose matrix at this temperature. On the other hand, for HMF and furfural, this model value is negative, meaning that the production of this compound only starts at more severe operational conditions. Using the inverse function of this model, given by eq. (4.1), and applying the parameter values previously described, it is possible to predict the severity factor value for which this type of degradation products start to be produced accordingly to this model. This equation is described by:

$$R_0(y) = \frac{ln(\frac{Plateau - y_0}{Plateau - y})}{K} \tag{4.1}$$

Applying eq. (4.1), HMF and furfural start to be produced at the severity factor of 5155.0 and 3948.2, respectively. In this work, that would have meant that HMF and furfural would only start to be produced at the conditions of around 160˚C for 90 min and 160˚C for 60 min, respectively. Observing the fig. 4.10, it is clear that, on those conditions, this degradation products are already being formed.

The relative percentage deviation (RPD) between the experimental concentration data and the values given by the different models are present in table 4.16.

Table 4.16: Predicted concentration of degradation products using the one-phase association and allosteric sigmoidal models with the severity factor (R_0). The values of relative percent different (RPD) are calculated between the experimental concentration data and the values given by each model expression. The negative values given by the one-phase association model, as the respective RPD, was not considered (n/d).

	One-phase association			RPD			Allosteric model			RPD		
R_0	Acetic Acid [mg/L]	HMF [mg/L]	Furfural [mg/L]	Acetic Acid (%)	HMF (%)	Furfural (%)	Acetic Acid [mg/L]	HMF [mg/L]	Furfural [mg/L]	Acetic Acid (%)	HMF (%)	Furfural (%)
2385.2	886.2	n/d	n/d	0.6	n/d	n/d	785.3	1.1	2.4	12.7	73	135
4109.3	1119.5	n/d	28.5	0.3	n/d	82	1129.5	3.6	17.6	1.1	51	118
5884.8	1335.4	7.6	326.8	1.5	33	67	1398.2	8.0	64.4	3.1	27	86
11131.5	1851.6	60.6	1062.3	4.3	59	45	1923.0	32.3	555.4	8.1	1.9	20
16525.3	2234.5	113.0	1634.4	3.3	40	18	2250.2	73.7	1511.8	2.6	2.4	9.8
23754.2	2584.1	179.7	2186.7	0.9	13	15	2531.9	147.9	2487.7	1.1	6.4	2.1
39628.2	2973.8	313.6	2863.7	0.9	4.4	13	2876.8	327.5	3102.7	4.2	0.0	5.4
64763.9	3159.5	494.3	3245.4	1.5	8.9	1.4	3138.7	526.5	3224.4	0.8	2.6	0.7
93064.7	3201.1	659.3	3353.4	0.8	3.9	7.6	3289.0	633.5	3243.1	1.9	0.1	4.2

All the computed models have given high correlation coefficients for every analysed by-product ($R^2 \geq 0.95$). However, the correlation of the model does not necessarily implicate an accurate prediction. For this reason, the prediction was analysed by having in consideration the relative percent deviation (RPD). When analysing the one-phase association model it was clear that the model gives a precise prediction of the acetic acid concentration, with a maximum deviation of 4.3%, regarding experimental data, however, the model produced negative concentration values for HMF and furfural concentrations for the least severe conditions. Even when these models values were positive, they presented relatively

high deviations for moderate working conditions. Considering the inhibition thresholds, the model gave inhibitory concentrations of acetic acid bellow the threshold, meaning, bellow 10 g/L [75]. Regarding furfural, the harshest condition that can be used, in other words, the one that did not surpass the HMF concentration threshold, was 160 ℃ for 90 min (severity factor value of 5885.8), either for both models or experimental data. For HMF, only the severest condition (200 ℃ and 90 min) surpassed the inhibitory concentration for this degradation product concentration. In the experimental data, the condition that surpassed first this concentration limit was 200 ℃ and 60 min (R_0 of 64763.9).

The goodness of fit was also assessed for the allosteric sigmoidal model. This model produced also high correlation coefficient ($R^2 \geq 0.98$), although this parameter decreased for acetic acid when compared with the previous one, therefore, it was observed a general increase on the relative deviation values using this model, exception made for the severity factor of 16525.3 and 64763.9. For HMF, the allosteric sigmoidal model not only gave positive concentration values, but also, produced lower relative deviations for the conditions with a severity factor higher than 5884.8. For the conditions that give a lower severity factor value, the relative percentage deviation value can reach the value of 75%. This high deviation its due to the HMF lower concentration value in this range of operations. An oscillation of 73% only means that the concentration value is between 0 and 2 mg/L, which is not significant in this project scope. For the case of furfural, it also produces positive values for all the conditions, however, it gives higher relative deviations when considering the severity factors of 4109.3 and 5885.8 when compared to the previous model. The lowest severe condition gives the highest relative deviation of all the data points analysed. Similarly, an oscillation of 135% only means that the furfural concentration value is between 0 and 5.6 mg/L, which is not significant in this project scope. Using this model, all the inhibitory concentration thresholds are in accordance with the experimental data values.

Based on the previous analysis, it was plotted in fig. 4.10 the models that presented a better goodness of fit to the experimental data, which means, one-phase association for the acetic acid modelling and allosteric sigmoidal model for the case of HMF and furfural.

Applying now the inverse equations for the remaining model, eq. (4.2), it is possible to analyse the prediction of the severity factor value for a given by-product concentration by the relative deviations between the value calculated using the trapezoidal method eq. (3.4), and the ones given by the inverse function of the models eq. (4.1) and eq. (4.2). The results are present in table 4.17.

$$R_0(y) = \left(-\frac{y-a}{b \cdot y}\right)^{-\frac{1}{h}} \tag{4.2}$$

The relative deviations, for each inverse model function, present a similar behavior to the one describe for the direct models. For acetic acid, the relative deviations for the one-phase association reach a maximum of 18% and for the allosteric sigmoidal this value reaches 22%. For HMF the relative deviations for the one-phase association reach a maximum of 77% and for the allosteric sigmoidal this value

Table 4.17: Severity factor (R_0) values given by the experimental data and the inverse equations of the developed models. The values of relative percent difference (RPD) are calculated between the experimental concentration data and the values given by each expression. The inverse model function does not apply when the concentration of degradation products surpasses the maximum value described by the parameters $Plateau$ and a, for the one-phase association and allosteric sigmoidal model, respectively, and so, this values described as (n/d) for the operational conditions where this applies.

	One-phase association R_0			RPD			Allosteric model R_0			RPD		
R_0	Acetic Acid	HMF	Furfural	Acetic Acid (%)	HMF (%)	Furfural (%)	Acetic Acid	HMF	Furfural	Acetic Acid (%)	HMF (%)	Furfural (%)
2385.2	2425.2	5379.1	4018.7	1.7	77	51	2864.3	3368.8	3736.4	18	34	44
4109.3	4087	5744.7	4335	0.5	33	5.3	4035	5200.8	5975.5	1.8	23	37
5884.8	6064.5	6170.6	4883.6	3.0	4.7	19	5574.7	6644.7	7646.7	5.4	12	26
11131.5	10217.8	8367	8194.5	8.6	28	30	9313.3	11230.2	11894.5	18	0.9	6.6
16525.3	17817.2	12644.4	13846.3	7.5	27	18	17779.5	16725	15744.5	7.3	1.2	4.8
23754.2	23134.2	21332.4	30418.1	2.6	11	25	24676.6	24627.2	24361.2	3.8	3.6	2.5
39628.2	41635	41409	69090.2	4.9	4.4	54	49245.2	39642.2	n/d	22	0.0	n/d
64763.9	54268.4	72081	59440	18	11	8.6	61434.9	67397.1	53906.6	5.3	4.0	18
93064.7	n/d	88329.2	51720.4	n/d	5.2	57	79116.9	93338.7	40081.9	16	0.3	80

reaches 34% and, for furfural, the relative deviations for the one-phase association reach a maximum of 57% and for the allosteric sigmoidal this value reaches 80%. The severity factor value changes with acetic acid concentration are better predicted by the first model, while the second one fits better for the HMF. For furfural, the errors given by the allosteric model, for the experimental severity factor values of 11131.5, 16525.3 and 2374.2 (corresponding to the holding temperature of 180˚C) are less than 7 %. However, the error for the other conditions is generally higher than the deviations given by the one-phase association model and, for the case of the experimental severity factor value of 39628.2 (200˚C for 30 min), the allosteric sinusoidal model failed to predict the severity factor since it already had surpassed the maximum value, given by the parameter a. This happened also when using the one-phase association model to predict the severity factor using the acetic acid concentration for the condition of experimental severity factor of 93064.7 (200˚C for 90 min), since this concentration is higher that the $Plateau$ value. Similarly to the previous goodness of fit assessment, for the models direct application, for both HMF and furfural, the severity factor values using the inverse function are more deviated for low severity conditions. However, for acetic acid and furfural, when the severity factor values are close to the maximum, given by the respective function parameters, the deviation tends to increase, even when using the more suitable inverse function of the model.

To evaluate the capacity of the inverse models equations to predict the maximum operational severity factor for a given degradation product threshold, it was taken the limit values using the previous works of Nigam (2001) [93] and Delgenes et al. (1996) [94], to compute the R_0 value with the limit for furfural (250 mg/L) and HMF (500 mg/L), respectively. Using the one-phase association model, the values of maximum severity obtained were 65646.5 for HMF and 5411.5 for furfural. In the first case, for HMF, the inverse model equation gives a higher severity factor threshold than the one considered by the experimental data (R_0 of 64763.9; for 200˚C and 60 min) and the most severe condition that can be applied, regarding only this degradation product, is the same. However, for furfural, the maximum

condition that does not surpass the furfural concentration threshold is now 160˚C for 60 min (R_0 of 4109.3). For the allosteric sigmoidal model, the values of maximum severity obtained were 60272.3 for HMF and 8682.1 for furfural. For HMF, the inverse model equation gives a lower severity factor threshold than the one considered by the experimental data (R_0 of 64763.9; for 200˚C and 60 min) and the most severe condition that can be applied, regarding only this degradation product, is now 200℃ and 30 min (R_0 of 39628.2). For furfural, the maximum condition that does not surpass the furfural concentration threshold is the same as for the experimental data, which means, 160˚C for 90 min (R_0 of 5884.8).

In order to assess the combinations of temperature and time that give the limiting severity factors, it was applied the severitity factor equation, eq. (3.1), in the holding phase. Since the temperature is constant in this phases, the integral function calculation results in:

$$R_0^{holding} = R_0 \times C_{holding} = t_{holding} \cdot exp\left(\frac{T_{holding} - T_{ref}}{\omega}\right) \tag{4.3}$$

In which, $R_0{}^{holding}$, $t_{holding}$ and $T_{holding}$ are the severity factor, time and temperatures of the holding phase. The holding phase contribution ($C_{holding}$) , previously described in table 4.3, was considered for the calculation of the $R_0{}^{holding}$ parameter. It was taken the mean value of the holding phases contributions, considering either the temperature or the holding time as fixed condition, in order to compute this parameter from the threshold of total severity factor values given by the inverse funtion of the considered model. The holding phase contribution values to the R_0 are present in table 4.18.

Table 4.18: Holding severity factor contribution mean value for the fixed operational conditions (either time or temperature).

time (min)	$C_{holding} = R_0{}^{holding}/R_0$	Temp (˚C)	$C_{holding} = R_0{}^{holding}/R_0$
30	0.77	160	0.88
60	0.89	180	0.86
90	0.91	200	0.83

It was calculated the threshold of holding temperature, by imposing a holding time. The computed holding temperature values are present in the table 4.19.

Similarly, it was calculated the threshold of holding time, by imposing a holding temperatue. The computed holding time values are present in table 4.20.

Table 4.19: Threshold of operational conditions and parameters, fixating the holding time, using the severity factor given by the one-phase association and allosteric sigmoidal inverse functions.

	One-phase association			
	R_0	$R_0^{holding}$	Fixed time (min)	Temp (˚C)
HMF	65646.5	50683.5	30	210
		58555.4	60	202
		59440.7	90	196
furfural	5411.5	4178.1	30	173
		4827.0	60	165
		4900.0	90	159
	Allosteric sigmoidal			
	R_0	$R_0^{holding}$	Fixed time (min)	Temp (˚C)
HMF	60272.3	46534.2	30	208
		53761.7	60	200
		54574.6	90	195
furfural	8682.1	6703.1	30	180
		7744.2	60	172
		7861.3	90	166

Table 4.20: Threshold of operational conditions and parameters, fixating the holding temperature, using the severity factor given by the one-phase association and allosteric sigmoidal inverse functions.

	One-phase association			
	R_0	$R_0^{holding}$	time (min)	Fixed Temp (˚C)
HMF	65646.5	57498.9	984	160
		56602.9	250	180
		54419.1	62	200
furfural	5411.5	4739.9	81	160
		4666.0	21	180
		4486.0	5	200
	Allosteric sigmoidal			
	R_0	$R_0^{holding}$	time (min)	Fixed Temp (˚C)
HMF	60272.3	52791.7	904	160
		51969.0	229	180
		49964.0	57	200
furfural	8682.1	7604.5	130	160
		7486.0	33	180
		7197.2	8	200

4.4.3 Lignin concentration analysis

The main goal of using LHW is to extract hemicellulosic sugars while maintaining, as much as possible, the other value-added components structure, such as lignin. If substantial lignin solubilization takes place in this pretreatment step, a further lignin extraction method, such as organosolv, might not be, neither efficient, nor even economically viable. Therefore, the goal regarding lignin, should be to maintain, as maximum as possible, this component in the solid fraction; in other words, avoid lignin solubilization and solubilization into the liquid stream. Also, it is in this work' scope to assess whether there is a

critical point in lignin solubilization when increasing the severity factor. The AIL, ASL and total lignin concentration profiles are present in fig. 4.11.

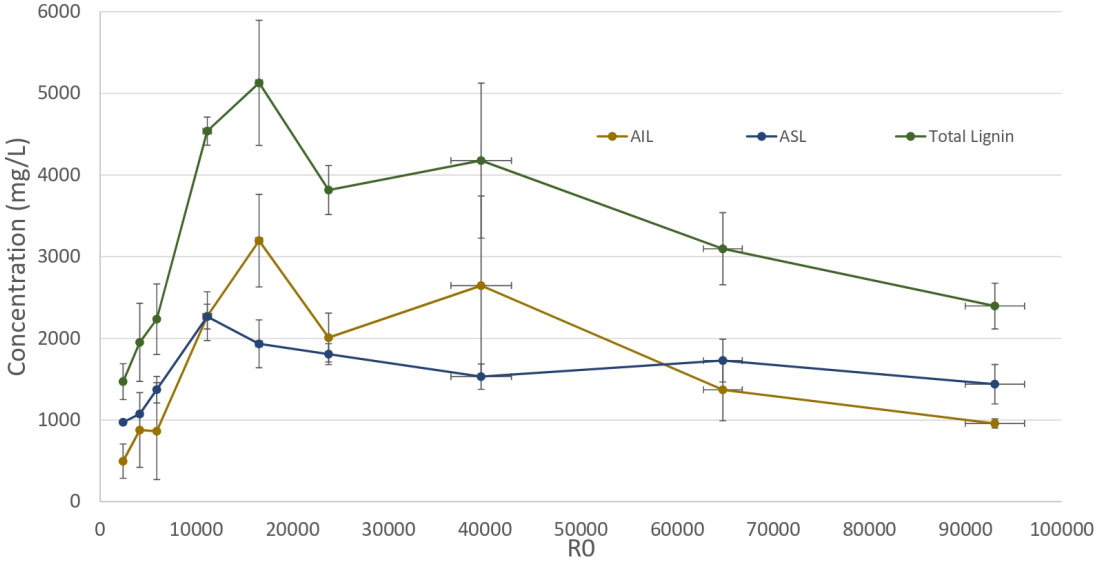

Figure 4.11: Profile of acid insoluble lignin (AIL), acid soluble lignin (ASL), total solubilized lignin (AIL + ASL) concentration, with the severity factor. All the points have their respective standard deviation bars.

This maximum point of lignin extraction occurs at the severity factor of 16525.3 (180℃ and 60 min holding conditions). After such conditions it is correct to state that the extracted lignin undergoes significant disintegration reaching, for the highest severity factor value, approximately 50% of the maximum total lignin extraction value. When analysis the lignin content by the Klason method (see section 3.3.5) it is clear that the total lignin concentration suffers a significant increase, about 2 times in the sum value of acid soluble and acid insoluble lignin, when increasing the severity factor from 5884.8 to 11131.5, which corresponds to an increase in the temperature of operation from 160 ℃ to 180ºC. For this project purposes, there is no interest on this increase in lignin solubilization into the liquid fraction since this sugar solution will be used for valorisation purposes, such as fermentations, which will not use lignin. Following this criterium, it is recomended to use a less harsh temperature than 180℃ to avoid an increase in lignin convertion.

The lignin percentage of convertion was calculated in order to study the level of lignin solubilization and how much of this component is still in the solid fraction, that resulted from the applied pretreatment,

and that is still available for extraction steps for further valorisation in the biomass refinery context. This values are described in table 4.21.

Table 4.21: Lignin conversion regarding structural lignin and their respective standard deviation and error values.

R_0	Convertion (%)			Error (%)
2385.2	10.1	±	1.3	13.3
4109.3	13.4	±	2.8	20.7
5884.8	15.3	±	2.5	16.7
11131.5	31	±	1.9	6.2
16525.3	35	±	4.7	13.3
23754.2	26.1	±	2.2	8.4
39628.2	28.6	±	5.5	19.3
64763.9	21.2	±	1.5	7.0
93064.7	16.4	±	1.8	11.0

Considering that the structural lignin content in the raw-material was 16.16 ± 0.87 wt%, it was calculated the lignin conversion using eq. (3.9) for each imposed severity conditions. The highest convertion percentage was obtained at 180℃ and 60 min, in conformity with the highest concentration of solubilized lignin. At this point and considering the worst case scenario, the remaining lignin in the solid phase could only be around 60 % of the initial lignin. In contrast, if a more moderate set of conditions is applied, as, for example, the threshold of degradation products conditions previously described (160 ℃ and 90 min) the amount of structural lignin present in the solid stream is a robust 82 % of the initial amount, having a conservative estimation that considers the statistical error. It is possible to decrease even more the conversion of lignin, using the less severe conditions of all, resulting in a 89 % lignin content in the solid stream, however, the success in the overall biorefinery goals, such as sugar production, could be compromised, and so not justifying an addition of just 7 % in this lignin content.

In order to modulate the total lignin profile with the severity factor, using eq. (3.5), it was assessed how the experimental data fitted the polynomial models. The parameters for modeling total lignin are described in table 4.22 as well as the respective correlation coefficient (R^2 and the severity factor domain of application.

Table 4.22: Lignin modeling parameters and the respective correlation coefficient (R^2) and the severity factor domain of application.

	a_4	a_3	a_2	a_1	a_0	R^2	Domain
Total Lignin	-1.21E-18	2.64E-13	-1.94E-08	0.000522	0.22	0.8559	[2385.2;93064.7]

The correlation coefficient (R^2) is 0.86 which indicates a reasonable fitting between the experimental values and the predicted model concentration, in all of the domain of experimentation. Computing the eq. (3.5) with the parameters described in table 4.21, it is given the predicted value of total lignin

concentrations, depending on the severity factor. The concentration values, as the respective relative percent deviation, are described in table 4.23.

Table 4.23: Lignin modeling and the respective relative percent deviation (RPD).

R_0	Lignin (mg/L)	RPD
2385.2	1358.3	7.9
4109.3	2055.4	5.2
5884.8	2672.4	18
11131.5	3972.3	13
16525.3	4649.5	9.8
23754.2	4826.3	23
39628.2	3885.5	7.2
64763.9	3082.8	0.5
93064.7	2802.9	16

Analysing the deviation values, it is observed that, despite of the correlation coefficient being lower that the ones previously described, the maximum of RPD is around 23 %. Although it is still a high percentage of deviation, it appears that the model predicts better lignin concentrations, at least, when considering data points in the neighbourhood of the experimental data. A more extended assessment should make this RPD values decrease and even increase the correlation coefficient.

4.5 Components simultaneous analysis

Since this work objective is to study sugar production in a biomass refinery context, an integrated analysis of all the assessed components must be made in order to chose the best fitting operational condition to use in larger scale applications. The mean value of all this components is stated in table 4.24.

Table 4.24: Analysed components concentration (mean value) for all experimental conditions.

			Degradation Products			**Monomeric Sugars**			**Total Sugars**			**Lignin**		
Temp. (℃)	Time (min)	Ro	Acetic Acid (mg/L)	HMF (mg/L)	Furfural (mg/L)	Mono C5 (mg/L)	Mono C6 (mg/L)	Mono Sugars (mg/L)	Total C5 (mg/L)	Total C6 (mg/L)	Total Sugars (mg/L)	AIL (mg/L)	ASL (mg/L)	Total Lignin (mg/L)
160	30	2385.2	891.8	2.3	12.5	480.0	91.3	571.3	3133.0	1564.1	4697.1	496.0	973.4	1469.4
	60	4109.3	1116.7	6.1	68.0	687.4	106.3	793.7	6990.3	2283.4	9273.7	876.9	1073.9	1950.9
	90	5884.8	1356.0	10.5	162.0	851.4	125.0	976.4	9562.1	2456.9	12018.9	863.5	1370.8	2234.2
180	30	11131.5	1773.3	32.9	675.6	1156.5	178.2	1334.8	9775.3	2147.8	11923.1	2271.1	2266.1	4537.2
	60	16525.3	2308.9	75.5	1370.5	2620.1	325.5	2945.6	10149.3	2371.0	12520.3	3196.0	1932.5	5128.5
	90	23754.2	2559.9	157.8	2540.0	2976.0	416.5	3392.5	8115.3	2442.6	10558.0	2009.1	1806.2	3815.3
200	30	39628.2	3001.2	327.6	3273.8	1396.8	541.3	1938.0	1402.8	1363.1	2765.9	2646.0	1530.5	4176.5
	60	64763.9	3113.9	540.5	3200.5	120.4	304.0	424.4	172.1	754.5	926.7	1369.5	1727.4	3097.0
	90	93064.7	3226.0	634.2	3108.4	41.0	159.5	200.6	77.0	437.2	514.2	957.2	1438.1	2395.3

This pretreatment method was able to solubilize a maximum monomeric sugars concentration of 3.4 g/L. In the work of Beisl et al. (2019) [77], a concentration of 1.4 g/L of monomeic sugars was not enough to fulfil the fermentation needs of *S. acidocaldarius*. After a concentration step, the same authors reported a 7.0 g/L concentration of total monomeric sugars, although in different C5/C6 ratios, as described before. Applying a similar concentration procedure to the sugar solution obtained, in this work,

at 180˚ for 90 min, would increase all of the components concentration, including degradation products, which, as described before, is inhibitory of fermentation procedures. However, when looking at the total sugar concentration values, it is observed that the maximum (12.5 g/L) is obtained at 180˚C and 60 min, without any concentration steps, which is close to the total sugar concentration stated by Beisl et al. (13.7 g/L), but in this case, after a concentration procedure. Nevertheless, for this condition, the degradation products threshold was already surpassed. The limiting concentration value considered in this work, as stated before, only allows the implementation of sugar solutions obtained from an extraction procedure with a severity factor of 5884.8, or less. The total sugar concentration at this severity factor (12.0 g/L) is around 96 % of the maximum total sugar concentrations that can be obtained and around 88 % of the concentration stated by Beisl et al. (2020), which means that this procedure it is close to fulfil this microorganism sugar requirments, since oligomeric sugars are used as substrate for some microorganisms (namely fungi), which includes *S. acidocaldarius*. The solubilized lignin, applying this pretreatment conditions (160˚C for 90 min), is around half of the maximum value for this work, which means, that lignin conversion is being kept to a minimum, as much as possible, and the solid fraction is still viable for further delignification processes, as described in the previous section.

This allows the statement that the best fitting conditions, of the ones that have been subjected to analysis, is to maintain a reaction temperature of 160˚C for 90 min.

5

Conclusions and further Perspectives

Contents

The use of wheat straw as lignocellulosic raw-material for sugar production and further valorization in the biomass refinery context has been proven to be effective. This work assessment has successfully fractioned the LCB component of interest while maintaining the remaining valiable structure largely unscattered, by using an highly available agro-industrial residue that, due to its natural recalcitrance, presents significant disposable challenges in its source. It was proven that this secondary product of wheat crops can be reused into the biotechnology concept and it has open the spectrum of biobased substances production using a more sustainable process than the ones currently being used in classical chemical industry.

5.1 Conclusions

This work main goal was subjecting wheat straw to a pretreatment method for hemicellulosic sugar production while evaluating, at the same time, the extraction of other main lignocellulosic components, in terms of concentration and selectivity, with changes the operational conditions.

Several pretreatments are presented in literature, but the one that is more suitable for hemicellulose sugar extraction, with low degradation of lignin and cellulose, inhibitory products formation within fixed thresholds and no use of environmental dangerous and expensive reagents was the autohydrolysis (liquid hot water) method.

Changing the method's aggressiveness towards the raw-material, meaning, manipulating the temperature and holding time set-up conditions, allowed a wide assessment of the several LCB components concentration profiles with the severity factor parameter (R_0). The first prominent conclusion was that the conditions that used a holding temperature of 200℃ are not suitable for this project purposes. The minimum holding time of this set of experiments, 30 minutes, for this temperature, not only gave an excess in degradation products concentrations provoked by the degradation of the extracted sugars (decrease in sugar production when comparing to less harsh conditions) but also significantly deconstructed cellulose and lignin, decreasing its value.

The optimal operational condition, that fits the biorefinery's objectives, balances the lowest holding temperature studied with the highest reaction duration, giving the set of 160 ℃ and 90 min for holding conditions. This conditions not only ensures degradation products concentration within the imposed thresholds but also give the highest concentration of oligosacharides, for both C5 and C6 sugars (around 11 g/L in total). For total sugars, this conditions produce also the maximum of concentration value in the case of C6, while for C5, this condition produces, 94% of the maximal condition (180 ℃ and 60 min). The maximum condition of total C5 sugars surpass the degradation products concentration limit, so, since the diference in the pentoses production concentration is only 6%, the sugar production objective is not compromised if the degradation products threshold is considered.

Regarding monosugars production, choosing the limit condition of 160℃ and 90 min operational conditions, gives only 29% and 23% of the maximum concentration for C5 and C6 single-molecule sugars, respectively. Sugar demands often varies depending on the application, however, if the goal is using a highest monomeric sugar's concentration, further LCB hydrolization might be needed. Previous works have reported that both concentrations, 1.0 g/L of monomeric sugars and 12.0 g/L of total sugars, obtained using this conditions, can be applied for further fermentation and consequent valorization, since this sugar values are comparable to the ones used in culture medium, for substrate concentration, in previous works. Since an increase in the severity factor is not possible due to the formation of degradation products, another pretreatment step can be added to the biorefinery extraction procedures, so, at least, the oligomers already solubilized could be further hydrolyzed into monomers, without the danger of lignin and cellulose destruction since this following step can be only applied to liquid stream after a moderate LHW fractioning step.

Regarding lignin solubilization, an increase in this components concentration in the liquid stream was observed with the increase in the severity factor until it reached its maximum. The use of 160℃ as holding temperature, for any holding time, not only meets the criterium for sugars and pretreatment degradation products but also sustain the lowest level of lignin deconstruction by side reactions. Although literature as presented some previous works in this area, specially focused on sugars and degradation products, it was not easy to find abundant lignin assessments for the remaining liquid fraction, which constitutes a new approach, described in this report, when analysing the amount of lignin that is being solubilized into the liquors. In this work, it is important to assess the amount of lignin that it is being transferred into the liquid stream, so a decision can be made regarding further delignification steps and their cost-effectiveness. As severity reached the value of 16525.3 (180℃ for 60 min), lignin has become more and more soluble and transferred into the liquid stream, reaching the maximum value at this condition. After this point, for more higher severity factor values, the lignin fraction has decreased in its concentration which can only mean that the rate of lignin degradation has surpassed the rate of lignin solubilization after the maximum point is reached. When implementing the working conditions that give a severity factor of 5884.8, it should be taken into consideration that 15% of the wheat straw lignin is being solubilized into the liquid stream, and, so, not being used for further valorization in the solid stream.

It was studied the fitting of the experimental data of all components concentration profile with the severity factor, to empirical models, however, the only data sets that produced consistent models were the degradation products. All the other LCB components only produced a polynomial model due to its concentration profile behavior, mainly, caused by the contribution of different parameters such as several reactions occurring at the same time (balancing between solubilization/degradation of sugar or lignin) and change on sugar sources (from hemicellulose to cellulose).

5.2 Further perspectives

Although it has been proved that the liquid sugar stream obtained, using the severity factor of 5884.8, can be applied in several fermentation procedures, it is still needed to assess, before a higher scale implementation, if cellular growth is viable using this operational parameters. It might be the case that using a more susceptible microorganism to inhibitory compounds in fermentation steps that cellular growth is not achieved at all, for example, using *S. acidocaldarius*. Further ajustments, for example detoxification steps (for example distilation, dilution or activated carbon), might be necessary to achieve cellular growth.

Comparing the conditions of 160℃/90 min with 180℃/30 min it is observed an increase in the severity factor by 90%. Since, as presented in this report, the degradation products thresholds, for HMF and furfural, are in between this two experiments, it would be of interest to apply the same protocol again, however, converging in this severity range (between the R_0 gap of 5884.8 and 11131.5). It would be interesting to use the model obtained in this work to, at least, give a narrower range of severity factor values to work with, and then, compare the concentrations of degradation products obtained by HPLC analysis with the ones given by the experimental models in order to assess its applicability. The use of more data points could even produce a better fitting model for degradation products concentration profile with the R_0.

Despite of the harshest operational conditions (200ºC holding temperature) not being applied in the work context of sugar production, this study could not predict the concentration *plateau* for HMF, as it did for acetic acid and furfural. This was mainly because, for the highest severity factor, there was still C6 sugars (mainly from cellulose) within the solution in a significant concentration, and so, suitable for degradation. This degradation products concentration *plateau* might be interesting to assess not only to study the case of temperature control malfunction (and sudden increase) but also finding the technology maximum by-products capacity if the biomass refinery changes its purpose from sugar to inhibitory compounds production.

The lignin concentration in the optimal condition for sugar extraction (160℃ and 90 min) is around 44% of the maximum value (180℃ for 60 min), being one of the lowest concentrations obtained for this component. Since this indicates that the lignin fraction remains in the pretreated solids, another fractioning step, specialized in lignin removal, might be interesting to be used in those solids, maximizing the extraction of value-added products from this feedstock.

In this work, it was used an isothermal protocol, characterized by having a holding phase at a fixed temperature. If an non-isothermal protocol was to be applied, in order to maintain the same severity factor values, it would be necessary to increase the maximum inner product temperature. It would be of interest to assess not only the effect of higher temperatures than 200℃, on all the studied components and parameters, but also the if the autoclave reactor used in work can give the necessary heat supply

to achieve this goal.

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Appendix A

A.1 Sample preparation

This section presents the equations used to calculate the water to be added into the mixture in order to maintain a accurate liquid-to-solid ratio LSR of 11:1. Equation A.1 gives the definition on the weight of dry matter content per weight of wet wheat straw.

$$Dry\ matter\ content = \frac{Dry\ matter\ weight}{Wet\ matter\ weight} \tag{A.1}$$

Using the mass balance definition, subtracting the dry matter weight to the wet matter weight gives the raw-material water content, which is what is represented in eq. (A.2).

$$Water\ content = \frac{Dry\ matter\ weight}{Dry\ matter\ content} - Dry\ matter\ weight \tag{A.2}$$

Combining both eq. (A.1) and eq. (A.2) gives the following equation for the water content:

$$Water\ content = Dry\ matter\ weight \cdot (1 - Dry\ matter\ content) \tag{A.3}$$

To achieve a proper and accurate proportion between the liquid and solid content, the water to be added will be total water present in the mixture minus the water content. This is given by eq. (A.4).

$$Water\ to\ add = Total\ water - Water\ content \tag{A.4}$$

Combining both eq. (A.4) and eq. (A.3) the result is the following general equation for the water to be added to the mixture:

$$Water\ to\ add = Total\ water - Dry\ matter\ weight \cdot (1 - Dry\ matter\ content) \tag{A.5}$$

A.2 First law of thermodynamics

This section serves just to state the first law of thermodynamics in order to better understand how the temperature and pressure inside the autoclave during the reaction have influence on each other proportionally and how sudden changes in this parameters affect the reactor control. This law is represented by eq. (A.6).

$$PV = nRT \tag{A.6}$$

Where P is the system pressure, V is the volume of control, n is the number of mols inside the volume of control, R is the universal gas constant and T is the system temperature.

A.3 Statistical treatment formulae

The mean value is always calculated by the following:

$$x_{mean} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{A.7}$$

The standard deviation (σ) value is always calculated by:

$$\sigma = \sqrt{\frac{(\sum_{i=1}^{n} x_i - x_{mean})^2}{n}} \tag{A.8}$$

The error propagation for sums is described by eq. (A.9).

$$\sigma_{sum} = \sqrt{\sum_{i=1}^{n} \sigma_i^2} \tag{A.9}$$

The error propagation for division, if $R = \frac{a}{b}$, is described by:

$$\sigma_{div} = R_{mean} \cdot \sqrt{\left(\frac{\sigma_a}{a_{mean}}\right)^2 + \left(\frac{\sigma_b}{b_{mean}}\right)^2} \tag{A.10}$$

For all this previous equations, x_{mean} is the mean value of all x values in the set; n is the number of samples in set and x_i is a measured value from the set.

The relative percent difference (RPD) between two samples, use the following calculation:

$$RPD = \frac{|X_1 - X_2|}{X_{mean}} \times 100 \tag{A.11}$$

Where X_1 and X_2 are measured values and X_{mean} is the mean value of X_1 and X_2.

A.4 Maximum error using trapezoids rule

When using an approximate computational method, such as the trapezoidal rule, there is always an associated error, given by the subtraction of the real value with the one resulted from the methods application. It is expected that this difference tends to the null value when the number of sub-intervals of integration, meaning, in this case, the number of trapezoids, N. So it is correct to state that the total error of integration is given by the sum of all the errors for each sub-interval. Each trapezoid as a height, h, equal to the Δt value imposed in this work (see section 3.3.8). If it is assumed that the function $f(T(t))$ is at least a C^2 class in the domain [0,t_{op}], meaning that their first two derivative functions are continuous in this interval, the maximum error obtained using the trapezoids rule for the approximate computation of the severity factor, in the domain of the time of operation, $E_0^{t_{op}} f(T(t))$, can be expressed by the following

equation:

$$\left|E_0^{t_{op}} f(T(t))\right| \leq M \cdot N \cdot \frac{h^3}{12} = \frac{t_{op}}{12} \cdot M \cdot \Delta t^2 \ , \ for\ h = \Delta t = \frac{t_{op}}{N}\ and\ M = \max_{t\epsilon[0,t_{op}]} \left|f^{''}(T(t))\right| \quad \text{(A.12)}$$

The function $f(T(t))$, used in the severity factor calculation, is given by:

$$f(T(t)) = exp\left(\frac{T(t) - T_{ref}}{\omega}\right) \quad \text{(A.13)}$$

The first derivative of this function is stated in eq. (A.14).

$$f'(T(t)) = \frac{T'(t) \cdot exp\left(\frac{T(t)-T_{ref}}{\omega}\right)}{\omega} \quad \text{(A.14)}$$

The second derivative of the function $f(T(t))$ is described in eq. (A.15).

$$f''(T(t)) = exp\left(\frac{T(t) - T_{ref}}{\omega}\right) \cdot \left(\frac{T''(t)}{\omega} + \frac{(T'(t))^2}{\omega^2}\right) \quad \text{(A.15)}$$

In order to estimate the M, it was assessed with condition of temperature and time gave the maximum $f''(T(t))$. In the calculations of the first two derivative values it was used the mean value theorem (or Lagrange theorem) since continuity is ensured by the previous stated condition of being a C^2 class function in the domain [0,t_{op}]. The computational tool used in this assessment was the *Maximum* function of Microsoft ® Excel, which was useful to return the maximum error value, $E_0^{t_{op}} f(T(t))$, for each severity condition. Summarizing, the specific method error for each of the setup conditions, as well as the maximum method error, are present in table A.1, together with all the parameters necessary to preform this computation.

Table A.1: Major error parameters and its respective maximum error calculation (in module). In this calculations, the Δt parameter is always equal to 1/60, in minutes.

R_0	Temp. (℃)	time (min)	t_{op} (min)	T'(t) (℃/min)	T"(t) (℃/min^2)	M	$\left\|E_0^{t_{op}} f(T(t))\right\|$	Specific Error (%)	Maximum Error (%)
2385.2	160.9	38.6	55.8	-5.4	648	2599.3	3.4	0.1	7.0
4109.3	160.6	66.3	82.1	-9	756	3139.5	6.0	0.1	4.0
5884.8	160.1	44.1	113.3	-5.4	648	12546.8	32.9	0.6	2.8
11131.5	180.5	60.9	70.6	-9	648	11771.8	19.2	0.2	1.5
16525.3	180.3	68.4	91.3	-9	648	10807.9	22.8	0.1	1.0
23754.2	181.1	68.4	120.3	-9	648	10807.9	30.1	0.1	0.7
39628.2	203.3	56.5	74.5	0	648	48079.6	82.9	0.2	0.4
64763.9	199.0	39.1	102.2	0	-1176	65367.7	154.7	0.2	0.3
93064.7	200.3	117.0	136.4	5.4	-864	52614.7	166.1	0.2	0.2

It was observed that the maximum error of the method is always less that the fixed threshold of 8 % of the severity factor mean value, which proves that the trapezoids integrative method can be applied to

this parameter calculation for this experimental data.

When comparing the maximum method error to the severity factor standard deviations, the error is lesser than the standard deviation values for the severity condition of 180 ℃ and 30 min, or higher. On this cases, with a holding temperature of 160 ℃, the maximum method's error value is always one order of magnitude below the severity factor average. This higher percentage of error values in the conditions that have 160 ℃ as holding temperature are expected since the maximum error is estimated by considering the most severe set of temperature parameters for its calculation, meaning, for a temperature of 200 ℃ and 90 min. When analysing the methods error individually, the specific errors are always lower that the standard deviation, which gives confidence when using the trapezoids rule, since the contribution of this deviations to the error propagation will not be significant.

It is worthwhile to remark that applying the Lagrange method's also inputs some error on this assessment, however, the Lagrange methods error is within the order of magnitude of the step Δt used, which is 10^3 times lower than the maximum error value, and around 200 times lesser than the lowest specific method error, so the mean value theorem's error contribution is always neglectable in the final error result, either for the specific or maximum errors.

A.5 Standard solution preparation

During concentration determination procedures it is imperative to have a guideline that assesses the relationship between the sensors signal to the equipment and the substance of interest concentration value - the correlation curve. To achieve this characteristic curve, it must be prepared, with maximum accuracy, the solutions that are going to describe the signal behavior with changes in the concentration. The standards concentrations must be such that the following samples concentration values are between the lowest and the highest standards concentration values, meaning, that the samples are within the range of assessment.

In this section, it is described how the standard solutions are prepared, not only to assess the degradation products but also the ones used to the sugar characterization.

A.5.1 Degradation products standard solutions

The by-products analysed in the HPLC were 5-hydroxymetylfurfural (HMF), furfural (C_4H_3OCHO) and acetic acid (CH_3COOH). So is was added to a volumetric flask an analytical amount of each of this components in order to get an accurate concentration inside the standard solution. This first volumetric flask - the stock solution - will be used to prepare more diluted standard solutions, with dilution factors of 1, 2, 5, 20, 100, 500 and 1000. All of this components are unstable, namely, HMF. This substance gives a particular challenge since it is highly volatile at room temperature. To overcome this reproducibility

obstacle, for each run in the HPLC, a new stock standard solution must be made, in order to ensure that the assessment conditions are maintained.

It is presented in table table A.2 the weight of all analytical component needed to prepare the stock standard solution its information regarding, not only the supplier, but also its purity, and, the working volume of the standard stock solution volumetric flask.

Table A.2: Stock solution for HPLC reagents supplier and concentration. Weighted mass for each reagent and volumetric flask capacity.

Supplier	Reagents	Aim concentration (mg/L)	Weighted mass (mg/L)	Flask (L)	Final concentration (mg/L)
Sigma- Aldrich (99.7%)	HMF	302.9	159.6	0.5	319.2
Merck (96%)	Acetic Acid	2054.8	987.3	0.5	1974.6
Sigma- Aldrich (99%)	Furfural	791	419.2	0.5	838.4

Some factors must be taken into consideration when preparing this stock standard solution, namely:

- Due to the volatility of some components, the weight measurements may be compromised by the fast substance evaporation.
- Furfural has a low solubility in water at room temperature and it can adhere to the goblet measurement flask glass walls, if this type labware is used in this component the weighting step

To minimize this preparation obstacles, and, since HMF is the most unstable component, it was maintained in the fridge as long as possible, being the last to be added to the stock solution. Secondly, for the furfural, this component was added directly in the volumetric flask, already half full of water, to facilitate the component dilution. This protocol was also applied in the case of the acetic acid in order to expedite the transfer step into the stock solution volumetric flask being this a very volatile component. For last, it is worth to remark that since HMF is solid at 4 ℃ (fridge temperature), it is not possible to add this component directly into the volumetric flask and get a accurate weight measurement since it will dissolve into the solution. So a regular - and as fast as possible - weighting step in a goblet flask was performed, with the respective washing steps, when transferring the HMF into the volumetric flask, in order to avoid mass losses.

A.5.2 Sugar standard solutions

Similar to the previous subsection (appendix A.5.1), the SSS were made by dissolving the sugars of interest in analytical amounts for calibration purposes. In this case, a long-term stock sugar standard solution can be made and stored and low temperature (freezer at -18 ℃) since it is stable for 6 months. It

is also noted that none of the previous challenges described in terms of substance solubility is applied in this case since all the sugars are soluble in water at room temperature and stable in terms of degradation. This long-term stock solution was made in order to have the sugars of interest concentration (glucose, xylose, arabinose, galactose and mannose) of 1000 mg/L of each.

This stock solution was used to prepare more diluted SSS to be used for calibration purposes on both types of sugar assessments. The dilutions were preformed in order to get final solutions concentrations of 1, 5, 10, 25, 50 mg/L of each sugar of interest. This step was made in duplicate, one set of diluted solutions to be used for monomeric sugar assessment and other to be applied in the total sugars quantification (see section 3.3.7).

Finally, focusing now on the seconds - that that use SRS as calibration reference - it was added (H_2SO_4) 4 % to the various previously produced diluted solutions, in order that each of the those were *diluted* with acid on a factor of 1:2, concluding the preparation of the SRS. It is worth to remark that this SRS solutions - after the addition of acid - are not stable in a long term perspective when compared to the SSS. So a new set of SRS must be conducted when preforming another assessment run, similarly to the appendix A.5.1.

B

Appendix B

B.1 Obtained values and statistical treatment

This appendix serves to report the experimental values obtained for each analysis that was performed in this work, as well as the statistical treatment done to the triplicates, meaning, the mean value and the standard deviation for each assessment.

Table B.1: Obtained values for each experimental run. AIL - Acid Insoluble Lignin; ASL - Acid Soluble Lignin.

Temp. (°C)	Aim hold time (min)	R_0	holding time (min)	Acetic Acid (mg/L)	HMF (mg/L)	Furfural (mg/L)	Total Deg. Products (mg/L)	AIL (mg/L)	ASL (mg/L)	Total Lignin (mg/L)
160	30	2489.0	30.3	980.9	4.1	16.8	1001.8	267.4	966.8	1234.2
		2397.5	30.7	854.6	1.5	13.1	869.2	539.9	964.8	1504.6
		2269.2	30.9	840.0	1.3	7.6	849.0	680.7	988.6	1669.3
	60	4143.6	61.5	1128.3	7.8	71.3	1207.4	1357.7	1084.6	2442.4
		4092.7	60.7	1108.2	5.5	69.1	1182.9	826.0	1097.4	1923.4
		4091.6	60.9	1113.6	5.0	63.5	1182.0	447.1	1039.8	1486.9
	90	6082.9	90.2	1375.7	11.7	181.3	1568.7	618.5	1435.8	2054.3
		5750.1	90.0	1330.9	10.0	147.6	1488.6	431.3	1490.3	1921.6
		5821.3	90.5	1361.3	9.8	157.0	1528.2	1540.7	1186.1	2726.8
180	30	11530.6	32.5	1824.7	35.3	736.1	2596.1	2066.5	2302.2	4368.7
		11336.5	31.1	1777.1	34.0	696.3	2507.4	2133.0	2396.7	4529.7
		10527.5	31.2	1717.9	29.5	594.4	2341.9	2613.7	2099.4	4713.1
	60	17018.0	61.0	2480.3	85.2	1580.2	4145.7	3615.6	2268.4	5884.0
		16262.5	60.8	2232.7	70.6	1264.7	3568.0	3421.3	1727.2	5148.5
		16295.5	60.2	2213.7	70.7	1266.4	3550.9	2551.1	1801.8	4352.9
	90	23719.6	92.0	2554.7	157.5	2501.0	5213.2	1923.8	1673.8	3597.7
		23491.9	90.4	2595.3	157.5	2581.2	5334.1	1761.6	1929.1	3690.8
		24051.2	91.0	2529.5	158.2	2538.0	5225.7	2341.9	1815.5	4157.4
200	30	44070.5	30.9	3049.9	365.0	3249.8	6664.7	1417.6	1708.9	3126.5
		37726.9	30.1	3062.8	307.8	3400.5	6771.1	2990.0	1438.2	4428.2
		37087.1	30.8	2890.9	310.0	3171.0	6371.9	3530.3	1444.3	4974.6
	60	62365.0	60.3	3114.9	539.1	3213.0	6867.0	664.2	1597.5	2261.6
		64641.6	60.9	3079.0	528.1	3281.8	6889.0	1526.4	1572.3	3098.7
		67285.0	60.3	3147.6	554.3	3106.7	6808.6	1400.1	1523.5	2923.6
	90	91377.7	90.3	3258.2	627.7	3113.8	6999.7	905.3	1422.1	2327.4
		90428.3	89.9	3153.5	613.1	3207.3	6973.8	1016.5	1687.1	2703.6
		97388.2	81.1	3266.2	661.7	3004.2	6932.2	949.9	1205.1	2154.9

Table B.3: Obtained values for each experimental run. C5 - Pentoses; C6 - hexoses.

Temp. (°C)	Aim hold time (min)	R_0	Total Arabinose (mg/L)	Total Galactose (mg/L)	Total Glucose (mg/L)	Total Xylose (mg/L)	Total Mannose (mg/L)	Total C5 (mg/L)	Total C6 (mg/L)	Total Sugars (mg/L)
160	30	2489.0	1039.6	399.8	1005.6	2296.1	174.8	3335.7	1580.2	4915.9
		2397.5	987.9	385.1	1033.0	2213.1	167.3	3200.9	1585.5	4786.4
		2269.2	933.5	370.3	1002.4	1928.9	153.7	2862.4	1526.5	4388.9
	60	4143.6	1288.3	552.8	1199.1	5085.5	298.4	6373.7	2050.4	8424.1
		4092.7	1631.9	679.7	1484.9	6160.7	373.7	7792.7	2538.3	10331.0
		4091.6	1474.2	615.1	1314.4	5330.4	332.0	6804.5	2261.5	9066.0
	90	6082.9	1334.2	588.2	1265.7	7521.2	337.6	8855.4	2191.4	11046.9
		5750.1	1659.4	733.3	1588.2	8714.6	403.0	10374.0	2724.5	13098.5
		5821.3	1502.2	658.6	1438.1	7954.6	357.9	9456.8	2454.6	11911.3
180	30	11530.6	884.8	565.2	1229.4	8912.8	292.3	9797.6	2086.9	11884.4
		11336.5	904.9	582.5	1294.7	8950.6	306.5	9855.6	2183.7	12039.2
		10527.5	978.8	593.0	1267.0	8694.0	312.8	9672.8	2172.8	11845.5
	60	17018.0	456.2	429.7	1683.1	9913.3	349.1	10369.5	2461.9	12831.4
		16262.5	478.5	407.0	1569.3	9473.2	298.9	9951.7	2275.2	12226.9
		16295.5	479.0	410.9	1610.3	9647.7	354.7	10126.7	2375.9	12502.6
	90	23719.6	247.0	428.4	1661.4	7849.2	355.7	8096.2	2445.5	10541.6
		23491.9	217.7	374.4	1480.0	7181.8	322.3	7399.5	2176.6	9576.1
		24051.2	276.4	474.0	1824.5	8573.9	407.4	8850.3	2705.9	11556.2
200	30	44070.5	29.2	121.1	1048.0	773.2	133.1	802.4	1302.3	2104.6
		37726.9	67.8	167.4	1017.4	1595.6	159.0	1663.4	1343.9	3007.3
		37087.1	78.3	130.6	1180.0	1664.5	132.5	1742.8	1443.1	3185.9
	60	62365.0	0.0	49.7	642.4	167.1	72.3	167.1	764.3	931.4
		64641.6	0.0	47.8	666.8	186.4	74.4	186.4	788.9	975.4
		67285.0	0.0	42.3	610.6	162.9	57.4	162.9	710.3	873.2
	90	91377.7	0.0	16.9	373.4	75.3	28.4	75.3	418.6	493.9
		90428.3	0.0	19.4	383.5	80.8	28.4	80.8	431.2	512.0
		97388.2	0.0	9.6	421.7	74.8	30.6	74.8	461.9	536.7

Table B.4: Average and standard deviation values for each set of operational conditions.

R_0			Acetic Acid (mg/L)			HMF (mg/L)			Furfural (mg/L)			Total Deg. Products (mg/L)		
2385.2	±	90.1	891.8	±	63.2	2.3	±	1.3	12.5	±	3.8	906.7	±	63.4
4109.3	±	24.2	1116.7	±	8.5	6.1	±	1.2	68.0	±	3.3	1190.8	±	9.2
5884.8	±	143.1	1356.0	±	18.7	10.5	±	0.8	162.0	±	14.2	1528.5	±	23.5
11131.5	±	434.4	1773.3	±	43.7	32.9	±	2.5	675.6	±	59.7	2481.8	±	74.0
16525.3	±	348.6	2308.9	±	121.4	75.5	±	6.9	1370.5	±	148.3	3754.9	±	191.8
23754.2	±	229.6	2559.9	±	27.1	157.8	±	0.3	2540.0	±	32.8	5257.6	±	42.6
39628.2	±	3152.0	3001.2	±	78.2	327.6	±	26.4	3273.8	±	95.2	6602.6	±	126.0
64763.9	±	2010.5	3113.9	±	28.0	540.5	±	10.8	3200.5	±	72.0	6854.9	±	78.0
93064.7	±	3081.6	3226.0	±	51.4	634.2	±	20.4	3108.4	±	83.0	6968.6	±	99.7

Table B.5: Average and standard deviation values for each set of operational conditions. Mono - Monomeric; C5 - Pentoses; C6 - hexoses.

R_0			Mono C5 (mg/L)			Mono C6 (mg/L)			Mono sugars (mg/L)		
2385.2	±	90.1	480.0	±	19.1	91.3	±	5.8	571.3	±	19.9
4109.3	±	24.2	687.4	±	6.8	106.3	±	0.6	793.7	±	6.9
5884.8	±	143.1	851.4	±	11.0	125.0	±	2.9	976.4	±	11.3
11131.5	±	434.4	1156.5	±	70.8	178.2	±	2.8	1334.8	±	70.8
16525.3	±	348.6	2620.1	±	80.5	325.5	±	4.0	2945.6	±	80.6
23754.2	±	229.6	2976.0	±	47.3	416.5	±	19.5	3392.5	±	51.2
39628.2	±	3152.0	1396.8	±	501.1	541.3	±	40.5	1938.0	±	502.8
64763.9	±	2010.5	120.4	±	12.8	304.0	±	10.2	424.4	±	16.3
93064.7	±	3081.6	41.0	±	5.0	159.5	±	7.0	200.6	±	8.6

Table B.6: Average and standard deviation values for each set of operational conditions. C5 - Pentoses; C6 - hexoses.

R_0			Total C5 (mg/L)			Total C6 (mg/L)			Total sugars (mg/L)		
2385.2	±	90.1	3133.0	±	163.1	1564.1	±	20.3	4697.1	±	164.3
4109.3	±	24.2	6990.3	±	481.1	2283.4	±	132.0	9273.7	±	498.9
5884.8	±	143.1	9562.1	±	510.8	2456.9	±	147.1	12018.9	±	531.6
11131.5	±	434.4	9775.3	±	120.1	2147.8	±	30.3	11923.1	±	123.9
16525.3	±	348.6	10149.3	±	181.2	2371.0	±	54.2	12520.3	±	189.2
23754.2	±	229.6	8115.3	±	569.0	2442.6	±	150.6	10558.0	±	588.6
39628.2	±	3152.0	1402.8	±	405.4	1363.1	±	74.4	2765.9	±	412.2
64763.9	±	2010.5	172.1	±	10.2	754.5	±	24.4	926.7	±	26.5
93064.7	±	3081.6	77.0	±	2.7	437.2	±	21.3	514.2	±	21.4

Table B.7: Average and standard deviation values for each set of operational conditions. Oligo - Oligosacharides; C5 - Pentoses; C6 - hexoses.

R_0			Oligo C5 (mg/L)			Oligo C6 (mg/L)			Total Oligo (mg/L)		
2385.2	±	90.1	2653.1	±	164.2	1472.7	±	21.1	4125.8	±	165.5
4109.3	±	24.2	6302.9	±	481.2	2177.1	±	132.0	8480.0	±	498.9
5884.8	±	143.1	8710.7	±	510.9	2331.9	±	147.1	11042.5	±	531.7
11131.5	±	434.4	8618.8	±	139.4	1969.5	±	30.5	10588.3	±	142.7
16525.3	±	348.6	7529.2	±	198.3	2045.5	±	54.4	9574.7	±	205.6
23754.2	±	229.6	5139.3	±	570.9	2026.2	±	151.9	7165.5	±	590.8
39628.2	±	3152.0	6.1	±	644.6	821.8	±	84.7	827.9	±	650.1
64763.9	±	2010.5	51.8	±	16.4	450.5	±	26.5	502.3	±	31.1
93064.7	±	3081.6	35.9	±	5.7	277.7	±	22.4	313.6	±	23.1

Table B.8: Average and standard deviation values for each set of operational conditions. AIL - Acid Insoluble Lignin; ASL - Acid Soluble Lignin.

R_0	AIL (mg/L)	ASL (mg/L)	Total Lignin (mg/L)
2385.2 ± 90.1	496.0 ± 210.1	973.4 ± 13.2	1469.4 ± 219.7
4109.3 ± 24.2	876.9 ± 457.4	1073.9 ± 30.3	1950.9 ± 478.3
5884.8 ± 143.1	863.5 ± 593.9	1370.8 ± 162.2	2234.2 ± 431.7
11131.5 ± 434.4	2271.1 ± 298.6	2266.1 ± 151.8	4537.2 ± 172.3
16525.3 ± 348.6	3196.0 ± 566.9	1932.5 ± 293.3	5128.5 ± 765.8
23754.2 ± 229.6	2009.1 ± 299.4	1806.2 ± 127.9	3815.3 ± 299.9
39628.2 ± 3152.0	2646.0 ± 1097.5	1530.5 ± 154.5	4176.5 ± 949.4
64763.9 ± 2010.5	1369.5 ± 380.3	1727.4 ± 262.8	3097.0 ± 441.5
93064.7 ± 3081.6	957.2 ± 56.0	1438.1 ± 241.4	2395.3 ± 280.6

Table B.9: Average and standard deviation values for each produced monomeric sugar (part 1) . Mono - Monomeric.

R_0	Mono Arabinose (%wt)	Err (%)	Mono Galactose (%wt)	Err (%)	Mono Glucose (%wt)	Err (%)
2385.2	0.4686 ± 0.0204	4.4	0.0200 ± 0.0008	4.0	0.0697 ± 0.0064	9.2
4109.3	0.6451 ± 0.0070	1.1	0.0401 ± 0.0006	1.6	0.0649 ± 0.0001	0.2
5884.8	0.7368 ± 0.0018	0.2	0.0616 ± 0.0015	2.5	0.0634 ± 0.0027	4.2
11131.5	0.5378 ± 0.0159	3.0	0.1081 ± 0.0022	2.0	0.0656 ± 0.0016	2.4
16525.3	0.4848 ± 0.0350	7.2	0.1744 ± 0.0021	1.2	0.1160 ± 0.0003	0.2
23754.2	0.2272 ± 0.0053	2.3	0.1721 ± 0.0055	3.2	0.1855 ± 0.0071	3.9
39628.2	0.0553 ± 0.0259	47	0.1363 ± 0.0340	25	0.3643 ± 0.0146	4.0
64763.9	0.0051 ± 0.0014	27	0.0374 ± 0.0033	8.8	0.2581 ± 0.0105	4.1
93064.7	0.0009 ± 0.0002	21	0.0073 ± 0.0020	27	0.1493 ± 0.0061	4.1

Table B.10: Average and standard deviation values for each produced monomeric sugar (part 2). Mono - Monomeric.

R_0	Mono Xylose (%wt)	Err (%)	Mono Mannose (%wt)	Err (%)	Mono Sugars (%wt)	Err (%)
2385.2	0.0641 ± 0.0056	8.8	0.0117 ± 0.0006	4.7	0.6341 ± 0.0221	3.5
4109.3	0.1166 ± 0.0031	2.6	0.0128 ± 0.0002	1.2	0.8795 ± 0.0077	0.9
5884.8	0.2054 ± 0.0120	5.8	0.0134 ± 0.0007	5.2	1.0805 ± 0.0125	1.2
11131.5	0.7394 ± 0.0765	10	0.0232 ± 0.0016	6.7	1.4740 ± 0.0782	5.3
16525.3	2.4077 ± 0.0813	3.4	0.0690 ± 0.0039	5.6	3.2519 ± 0.0887	2.7
23754.2	3.0629 ± 0.0520	1.7	0.1028 ± 0.0196	19	3.7506 ± 0.0565	2.0
39628.2	1.4914 ± 0.5542	37	0.0987 ± 0.0253	26	2.1460 ± 0.5566	26
64763.9	0.1283 ± 0.0141	11	0.0415 ± 0.0023	5.5	0.4704 ± 0.0181	3.8
93064.7	0.0446 ± 0.0055	12	0.0203 ± 0.0043	21	0.2224 ± 0.0095	4

Table B.11: Average and standard deviation values for each produced total sugar (part 1).

R_0	Total Arabinose (%wt)	Err (%)	Total Galactose (%wt)	Err (%)	Total Glucose (%wt)	Err (%)
2385.2	1.0955 ± 0.0480	4.4	0.427 ± 0.013	3.1	1.125 ± 0.015	1.4
4109.3	1.6231 ± 0.1558	9.6	0.682 ± 0.057	8.4	1.477 ± 0.130	8.8
5884.8	1.6585 ± 0.1471	8.9	0.730 ± 0.066	9.0	1.583 ± 0.146	9.2
11131.5	1.0191 ± 0.0447	4.4	0.641 ± 0.013	2.0	1.396 ± 0.030	2.1
16525.3	0.5202 ± 0.0118	2.3	0.459 ± 0.011	2.4	1.789 ± 0.052	2.9
23754.2	0.2731 ± 0.0265	9.7	0.470 ± 0.045	9.6	1.830 ± 0.156	8.5
39628.2	0.0647 ± 0.0234	36	0.155 ± 0.022	14	1.198 ± 0.078	6.5
64763.9	0 ± 0	-	0.052 ± 0.003	6.7	0.709 ± 0.025	3.6
93064.7	0 ± 0	-	0.017 ± 0.005	27	0.436 ± 0.023	5.3

Table B.12: Average and standard deviation values for each produced total sugar (part 2).

R_0	Total Xylose (%wt)	Err (%)	Total Mannose (%wt)	Err (%)	Total Sugars (%wt)	Err (%)
2385.2	2.382 ± 0.174	7.3	0.183 ± 0.010	5.3	5.2133 ± 0.1823	3.5
4109.3	6.123 ± 0.510	8.3	0.371 ± 0.034	9.2	10.2760 ± 0.5531	5.4
5884.8	8.924 ± 0.547	6.1	0.405 ± 0.030	7.5	13.3013 ± 0.5893	4.4
11131.5	9.776 ± 0.125	1.3	0.336 ± 0.009	2.8	13.1670 ± 0.1366	1.0
16525.3	10.684 ± 0.198	1.9	0.369 ± 0.028	7.5	13.8219 ± 0.2074	1.5
23754.2	8.699 ± 0.628	7.2	0.400 ± 0.039	9.7	11.6724 ± 0.6506	6
39628.2	1.489 ± 0.448	30	0.157 ± 0.014	8.7	3.0628 ± 0.4562	15
64763.9	0.191 ± 0.011	5.9	0.075 ± 0.008	11.1	1.0272 ± 0.0293	2.9
93064.7	0.085 ± 0.003	3.5	0.032 ± 0.001	3.6	0.5702 ± 0.0238	4

Table B.13: Average and standard deviation values for each monomeric sugar yield of production (part 1). Mono - Monomeric.

R_0	Mono Arabinose Yield (%wt)	Err (%)	Mono Galactose Yield (%wt)	Err (%)	Mono Glucose Yield (%wt)	Err (%)
2385.2	20.7812 ± 1.7211	8.3	2.8861 ± 0.1228	4.3	0.1907 ± 0.0206	10.8
4109.3	28.6081 ± 2.0395	7.1	5.7832 ± 0.1236	2.1	0.1777 ± 0.0101	6.0
5884.8	32.6763 ± 2.3037	7.0	8.8785 ± 0.2547	2.9	0.1734 ± 0.0123	7.0
11131.5	23.8498 ± 1.8227	7.6	15.5840 ± 0.3813	2.4	0.1794 ± 0.0111	6.2
16525.3	21.5024 ± 2.1698	10.1	25.1437 ± 0.4643	1.8	0.3174 ± 0.0181	5.7
23754.2	10.0757 ± 0.7473	7.0	24.8117 ± 0.8697	3.5	0.5076 ± 0.0349	7.0
39628.2	2.4515 ± 1.1610	47	19.6596 ± 4.9126	25	0.9967 ± 0.0693	6.9
64763.9	0.2270 ± 0.0629	28	5.3948 ± 0.4819	8.9	0.7062 ± 0.0494	7.0
93064.7	0.0392 ± 0.0088	22	1.0550 ± 0.2859	27	0.4085 ± 0.0286	7.0

Table B.14: Average and standard deviation values for each monomeric sugar yield of production (part 2). Mono - Monomeric.

R_0	Mono Xylose Yield (%wt)			Err (%)	Mono Mannose Yield (%wt)			Err (%)	Mono Sugar Yield (%wt)			Err (%)
2385.2	0.2762	±	0.0253	9.2	1.5633	±	0.1259	8.1	2.3576	±	0.0998	4.2
4109.3	0.5022	±	0.0185	3.7	1.7152	±	0.1136	7	3.2995	±	0.0801	2.4
5884.8	0.8845	±	0.0564	6.4	1.7935	±	0.1493	8.3	4.0620	±	0.1036	2.6
11131.5	3.1836	±	0.3394	10.7	3.1108	±	0.2917	9.4	5.5306	±	0.3312	6.0
16525.3	10.3664	±	0.4397	4.2	9.2569	±	0.7944	8.6	12.2978	±	0.4461	3.6
23754.2	13.1877	±	0.4057	3.1	13.8005	±	2.7757	20	14.1244	±	0.3838	2.7
39628.2	6.4213	±	2.3917	37	13.2492	±	3.5059	26	7.6478	±	2.1870	29
64763.9	0.5524	±	0.0623	11.3	5.5658	±	0.4756	9	1.4107	±	0.0805	5.7
93064.7	0.1921	±	0.0244	12.7	2.7265	±	0.6017	22.1	0.6443	±	0.0394	6.1

Table B.15: Average and standard deviation values for each total sugar yield of production (part 1).

R_0	Total Arabinose Yield (g/g)			Err (%)	Total Galactose Yield (%wt)			Err (%)	Total Glucose Yield (%wt)			Err (%)
2385.2	48.5835	±	4.0314	8.3	61.6348	±	2.1098	3.4	3.0783	±	0.1799	5.8
4109.3	71.9841	±	8.5700	11.9	98.4057	±	8.3983	8.5	4.0409	±	0.4238	10
5884.8	73.5535	±	8.3326	11.3	105.3300	±	9.5844	9.1	4.3321	±	0.4694	11
11131.5	45.1970	±	3.7502	8.3	92.3943	±	2.2383	2.4	3.8184	±	0.2316	6.1
16525.3	23.0719	±	1.7082	7.4	66.1976	±	1.8173	2.7	4.8960	±	0.3122	6.4
23754.2	12.1134	±	1.4523	12	67.8418	±	6.5579	9.7	5.0071	±	0.5120	10
39628.2	2.8694	±	1.0571	37	22.3091	±	3.2055	14	3.2778	±	0.2832	8.6
64763.9	0	±	0	-	7.4456	±	0.5123	6.9	1.9409	±	0.1305	6.7
93064.7	0	±	0	-	2.4429	±	0.6677	27	1.1921	±	0.0926	7.8

Table B.16: Average and standard deviation values for each total sugar yield of production (part 2).

R_0	Total Xylose Yield (%wt)			Err (%)	Total Mannose Yield (%wt)			Err (%)	Total Sugars Yield (%wt)			Err (%)
2385.2	10.2555	±	0.7958	7.8	24.6176	±	2.0633	8.4	18.2171	±	0.8242	4.5
4109.3	26.3619	±	2.2980	8.7	49.7648	±	5.6107	11	37.0597	±	2.2811	6.2
5884.8	38.4223	±	2.5526	6.6	54.3744	±	5.3910	9.9	48.6888	±	2.5075	5.1
11131.5	42.0915	±	1.2057	2.9	45.0230	±	3.1962	7.1	48.6101	±	1.2023	2.5
16525.3	46.0017	±	1.4564	3.2	49.5091	±	4.9212	9.9	50.8629	±	1.3808	2.7
23754.2	37.4533	±	2.8712	7.7	53.6701	±	6.2622	12	42.3198	±	2.6763	6.3
39628.2	6.4097	±	1.9365	30	21.0334	±	2.2906	11	10.0700	±	1.7937	18
64763.9	0.8216	±	0.0532	6.5	10.1128	±	1.3040	13	2.9506	±	0.1480	5.0
93064.7	0.3674	±	0.0160	4.4	4.3317	±	0.3230	7.5	1.6114	±	0.0945	5.9