INSTITUTO
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Universidade de Lisboa

BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC MATERIALS MIXTURES USING RECOMBINANT STRAINS OF *Saccharomyces cerevisiae*

Tese apresentada para obtenção do grau de Doutor em Engenharia do Ambiente

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To my daughter Maria Paula

Publications

The results of this thesis are/will be published as follows:

1. **T. Silva-Fernandes**, L. C. Duarte, F. Carvalheiro, S. Marques, M. C. Loureiro-Dias, C. Fonseca, F. Gírio; 2015. Biorefining strategy for maximal monosaccharide recovery from three different feedstocks: eucalyptus residues, wheat straw and olive tree pruning. Bioresource Technology. 183:203-212.

2. **T. Silva-Fernandes**, L. C. Duarte, F. Carvalheiro, M. C. Loureiro-Dias, C. Fonseca, F. Gírio; 2015. Hydrothermal pretreatment of several lignocellulosic mixtures containing wheat straw and two hardwood residues available in Southern Europe. Bioresource Technology. 183:213-220.

3. **T. Silva-Fernandes**, S. Marques, R. C. L. B. Rodrigues, M. C. Loureiro-Dias, C. Fonseca, F. Gírio. Optimization of glucose recovery by enzymatic hydrolysis of hydrothermally pretreated eucalyptus residues, wheat straw or olive tree pruning and their mixtures *[in prepraration]*

4. **T. Silva-Fernandes**, S. Marques, M. C. Loureiro-Dias, C. Fonseca, F. Gírio. Co-fermentation of glucose and xylose obtained from mixtures of eucalyptus residues, wheat straw and olive tree pruning hydrothermally pretreated at high solid loading with recombinant *Saccharomyces cerevisiae* BH42 *[in prepraration]*

“Todas as vitórias ocultam uma abdicação”

Simone de Beauvoir

(1958)

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Abstract

Bioethanol Production from Lignocellulosic Materials Mixtures Using Recombinant Strains of *Saccharomyces cerevisiae*

The use of mixtures of lignocellulosic materials is an important contribution to the sustainability of biorefineries since it may contribute to overcome problems related with individual feedstock availability, such as amount and seasonality. In this work, eucalyptus residues, wheat straw and olive tree pruning were studied towards their potential use in bioethanol biorefineries. These materials, largely available in the Southern Europe, can be considered complementary feedstock from the seasonal point of view. Moreover, the deployment of lignocellulosic ethanol technology depends on the full integration of the hemicellulosic fraction in the value-chain. Therefore, this work aims at an efficient conversion of both the cellulosic and the hemicellulosic fractions from lignocellulosic materials mixtures into bioethanol through the optimization and integration of pretreatment, enzymatic hydrolysis and fermentation stages under different configurations. Autohydrolysis has been studied as pretreatment method. Response surface methodology was used to optimize glucose recovery by enzymatic hydrolysis of the pretreated lignocellulosic materials. Finally, different fermentation strategies for the conversion of glucose and xylose were studied using a recombinant xylose-fermenting *Saccharomyces cerevisiae* strain. The feasibility of converting mixtures of lignocellulosic materials into bioethanol was demonstrated and this study is expected to contribute to its industrial implementation for second-generation (2G) bioethanol production.

Keywords

Bioethanol, xylose-fermenting *Saccharomyces cerevisiae*, enzymatic hydrolysis, hydrothermal treatment, mixtures of lignocellulosic materials.

Resumo

Produção de Bioetanol a partir de Misturas de Materiais Lenhocelulósicos usando Estirpes Recombinantes de *Saccharomyces cerevisiae*

O uso de misturas de materiais lenhocelulósicos é uma importante contribuição para a sustentabilidade das biorrefinarias, uma vez que pode minimizar problemas de disponibilidade de matéria-prima, como quantidade e sazonalidade. Neste trabalho, foram estudados resíduos de eucalipto, palha de trigo e podas de oliveira para utilização em biorrefinarias de bioetanol lenhocelulósico. Estes materiais, amplamente disponíveis no Sul da Europa, podem ser considerados complementares do ponto de vista sazonal. A implantação da tecnologia de etanol lenhocelulósico depende da integração da fração hemicelulósica na cadeia de valor. Assim, este trabalho visa a conversão eficiente das frações celulósica e hemicelulósica de misturas de materiais lenhocelulósicos em bioetanol, através da otimização e integração das etapas de pré-tratamento, hidrólise enzimática e fermentação em diferentes configurações. A auto-hidrólise foi estudada como método de pré-tratamento. Foi utilizada metodologia de superfície de resposta para otimizar a recuperação de glucose por hidrólise enzimática dos materiais lenhocelulósicos pré-tratados. Finalmente, foram estudadas diferentes estratégias de fermentação, para a conversão de glucose e xilose, usando uma estirpe de *Saccharomyces cerevisiae* recombinante fermentadora de xilose. Foi demonstrada a viabilidade de conversão de misturas de materiais lenhocelulósicos em bioetanol e pretende-se que este estudo contribua para a implementação industrial do bioetanol de segunda geração (2G).

Palavras-chave

Bioetanol, hidrólise enzimática, misturas de materiais lenhocelulósicos, *Saccharomyces cerevisiae* fermentadora de xilose, tratamento hidrotérmico.

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Thesis structure

This thesis comprises five Chapters whose contents are described below.

Chapter I contains a general introduction and provides the information considered essential to allow an understanding of the experimental work presented in the following chapters. In this Chapter is discussed the importance of biofuels with a special emphasis for 2G bioethanol. It also contains information about the feedstock materials used for the production of this biofuel with particular focus on eucalyptus residues, wheat straw and olive tree pruning, which are abundant lignocellulosic materials in Southern Europe countries. The main steps for 2G bioethanol production, pretreatment, enzymatic hydrolysis and fermentation are also discussed. Finally, this Chapter describes the technological challenges of 2G bioethanol biorefineries, and the innovation of this work, including the use of mixtures of lignocellulosic materials and the proposed objectives in this thesis.

The following three Chapters (II, III and IV) report the experimental work developed, each chapter focusing in one of the main steps of 2G bioethanol production from lignocellulosic materials: pretreatment, enzymatic hydrolysis and fermentation.

Chapter II refers to the pretreatment process of lignocellulosic materials and it is divided into two parts (A and B). Part A describes an integrated strategy for total sugar recovery from eucalyptus residues, wheat straw and olive tree pruning in a biorefinery framework. The autohydrolysis profile of each feedstock was performed in order to understand individual properties and their potential contribution in mixtures. The conditions of post-hydrolysis of the liquid fraction from autohydrolysis were optimized aiming maximal monosaccharides recovery. The solid fraction obtained from autohydrolysis was subject to enzymatic hydrolysis to assess digestibility of pretreated materials. The integrated biorefining strategy thus combine autohydrolysis, post-hydrolysis and enzymatic hydrolysis of individual feedstocks for maximal monossacharides recovery. Moreover, the mixture of lignocellulosic materials is preliminarily discussed in face of the results obtained with each individual feedstock. In part B, the processing of biomass mixtures containing eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) in different proportion (ER:WS:OP; 50:25:25; 25:50:25 and 25:25:50, represented by 50ER, 50WS and 50OP, respectively) is presented. The liquid and solid fraction recovered after autohydrolysis was characterized and the results are discussed based on the composition of the individual feedstock. The results were evaluated within a broad severity factor (Log R_0) range and an empirical model was used to optimize the pretreatment conditions for maximal saccharides recovery based on the chemical characterization of the liquid fraction. The post-hydrolysis condition optimized in part A was applied to obtain the maximal saccharides recovery from feedstock mixtures. The similarity between the composition of the mixtures represented by 50ER, 50WS and 50OP and the advantages of their use in multi-feedstock biorefineries are discussed.

In **Chapter III**, the enzymatic hydrolysis of the solid fraction of eucalyptus residues, wheat straw or olive tree pruning obtained from hydrothermal pretreatment is studied and statistically analysed for maximal glucose recovery. The employed strategy aimed the use of hydrolysis yields with individual feedstock to predict the results for mixtures. Central Composite Face Centred (CCFC) was used for experimental design with pretreatment temperature (210-230ºC), enzyme dosage (0.25-0.75 g/g_{solids}) (Celluclast 1.5L and Novozyme 188 cocktail) and solid loading (5-20% w/v) as the independent variables studied. The target of this study was to optimize the glucose concentration and yield from the solid fraction of pretreated materials. The developed models for each individual feedstock were used to provide results for different combinations of feedstock mixtures. The use of statistical modelling to optimize enzymatic hydrolysis of individual feedstock and their mixtures is discussed.

Chapter IV integrates fermentation with the results obtained for pretreatment and enzymatic hydrolysis (Chapters II and III). The study performed on this Chapter focus on upgrading the cellulosic and hemicellulosic fractions of lignocellulosic mixtures (50ER, 50WS and 50OP), at high solid loading (20% w/v), into 2G bioethanol. The solid and liquid fractions recovered after the autohydrolysis were subjected to simultaneous saccharification and co-fermantation (SSCF) and separated hydrolysis and co-fermentation (SHCF) using the recombinant yeast *Saccharomyces cerevisiae* BH42, and the results were compared with those obtained with the liquid fraction replaced by chemically defined liquid medium containing xylose. The process configurations are compared and discussed. The effect of potential inhibitors on enzymatic hydrolysis and yeast performances is also discussed in face of results obtained. Finally, the feasibility of the overall process for the conversion of different mixtures of ER, WS and OP into 2G bioethanol is discussed.

In the final **Chapter (V)** a general discussion is presented focusing in the utilization of feedstock mixtures as an attractive solution for 2G bioethanol biorefineries in regions of limited biomass availability. Finally, a perspective for future work is presented based on unanswered questions and on the novel contributions given by this thesis, towards the propagation of 2G bioethanol biorefineries based on mixtures of lignocellulosic materials.

CHAPTER

1

GENERAL INTRODUCTION

1.1 The importance of biofuels

The climate change, caused by the increasing greenhouse gases (GHG) emissions, the depletion of fossil fuel reserves and the security of energy supply are nowadays among the most critical societal concerns in the fields of environment and energy. Apart from the need of increasing energy savings and energy efficiency, the use alternative fuels is mandatory to replace part the consumption of fossil fuels. In this sense biofuels have been proposed as attractive alternative to petroleum-based fuels. They are based on biomass, which is abundant and, in many cases, untapped, making it a renewable and environmentally friendly fuel (Balat, 2011; Cardoso et al., 2012; Demirbas, 2009; Sarkar et al., 2012).

The use of biofuels can contribute to the mitigation of GHG emissions, improving air quality, provide a clean and therefore sustainable energy source, and boost rural economies. Large-scale production of biofuels offers an opportunity for certain developing countries to reduce their dependence on oil imports and to co-produce foods and fuels. In developed countries there is a growing trend towards employing modern technologies and efficient bioenergy conversion using a range of biofuels, which are becoming cost-wise competitive with fossil fuels (Balat, 2011; Demirbas, 2007). The required sources are geographically more evenly distributed than the fossil fuels; thus, the sources of energy will, to a larger extent, be domestic and provide security of supply (Hahn-Hägerdal et al., 2006; Petrova and Ivanova, 2010).

1.1.1 Biofuels in the transportation sector

Renewable liquid biofuels for transportation have recently attracted huge attention worldwide despite its large production and consumption in some countries like Brazil and USA. The special attention given to transport, has been due to the large energy consumption and significant pollution generated by this sector. Today, the transportation sector worldwide is almost entirely dependent on petroleum-based fuels and has been responsible for 60% of the world oil consumption (Tsita and Pilavachi, 2013). In 2008 the transportation sector accounted for about 22% of total world CO_2 emissions (Atabani et al., 2011). The world vehicles fleet is estimated to grow about 1.6 billion units in the next 15 years (Pielke, 2013) and this growth will affect the stability of ecosystems and global climate as well as global oil reserves.

Thus, many countries have implemented or are implementing programs for ethanol blending in gasoline. Moreover, many countries also have set national biofuels targets and are providing incentives and supports to accelerate the growth of bioenergy industry.

In the European Union (EU), in response to interest in biofuels, in 2003 the policy makers enacted the Directive on Renewable Energy (2003/30/EC) which established the non-mandatory incorporation of 5.75% biofuels by 2010 in the transport sector, with an interim target of 2% by 2005. Later this has been superseded by Directive in 2009 (2009/28/EC) which set a new mandatory target of 10% to be met with renewable sources by 2020. This directive lays down sustainability criteria for biofuels used to meet the target, including minimum GHG savings, exclusion of certain land types and assessment of impact of biofuel on food and social issues; it allows double counting of biofuels from wastes, residues and lignocellulosic materials for the purposes of meeting the target and incentives to electric vehicles powered from renewable sources (Smyth et al., 2010).

In the short term, biofuels are the only renewable resources that can address the transportation sector’s heavy dependence on oil without replacing the vehicle fleet (Yue et al., 2014).

1.2 Bioethanol: The renewable fuel most used worldwide in transport

Bioethanol is the most used liquid biofuel for transportation worldwide either as a fuel or as a gasoline enhancer (additive) and have been seen as the only possible option, in the short term, to partially replace the consumption of fossil fuels in the transport sector (Balat, 2009; Balat, 2011; Petrova and Ivanova, 2010). A significant decrease in GHG emission can be obtained with the bioethanol production and use on a life-cycle basis (Wang et al., 2007; Niven, 2005). Its low freezing point makes it suitable for use in cold climates and can be applied directly, as a replacer or additive, or in the form of partially renewable ETBE (ethyl tertiary butyl ether), which is an octane enhancer used in fuel blends currently produced by chemical synthesis from oil-derivatives. It can be blended with gasoline in any combination, and it is currently approved as a 5% or 10-20% blend for all vehicles (in Europe or USA and Brazil, respectively) and as an 85% blend or 100% (hydrated) for flex-fuel vehicles.

The use of bioethanol has some advantages. Firstly, it has a higher oxygen content that implies a less amount of additive required to enhance the level of octanes. The increased percentage of oxygen allows a better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO and aromatic compounds. Moreover, bioethanol has higher octane booster properties than some additives (e.g. MTBE), is not toxic and does not contaminate water sources (Song et al., 2006). Bioethanol flammability in air is also much lower than that of gasoline, which reduces the number and severity of vehicle fires (Dodic et al., 2009).

Bioethanol has already been introduced on a large scale in Brazil, in USA and can be one of the dominating renewable biofuels in the transport sector in European countries. This biofuel can be produced from different kinds of raw materials. Currently commercialized bioethanol, mainly in the USA and Brazil, is the so-called first-generation (1G), based on food crops. This technology uses sucrose or starch-containing materials (Balat, 2011; Sanchez and Cardona, 2008). Sugars, from sugarcane, sugar beets and sweet sorgum molasses can be directly fermented to bioethanol (Foteinis et al., 2011; Wang et al., 2014). Starches, from corn, wheat, cassava or other cereal must be hydrolyzed to fermentable sugars by the action of enzymes prior to fermentation into bioethanol (Lin and Tanaka, 2006; Patni et al., 2013; Shanavas et al., 2011; Yue et al., 2014). Alternatively, the production of bioethanol from lignocellulosic materials has been proposed (Christakopoulos et al., 1989; Tran and

Chambers, 1986; Wyman et al., 1992). The use of these raw materials is an alternative to ethanol 1G, and is considered second-generation biofuel technology (2G).

1.2.1 The production of 2G bioethanol

Although the 1G technology is very well established, the 2G technology has attracted great interest worldwide. The raw material required for 2G bioethanol production, lignocellulosic materials, is abundant and less expensive than conventional agricultural feedstock (grain), furthermore, the price of the raw materials used on 1G technology is also highly volatile, which can highly affect the production costs of bioethanol (Sims et al., 2008).

Biomass-to-bioethanol conversion technologies can be classified into two categories, namely thermochemical and biochemical (Balat et al., 2008; Foust et al., 2009; Yue et al., 2014). There are two bioethanol production processes that currently employ thermochemical reactions. The first system is actually a hybrid thermochemical and biological system. Biomass materials are first thermochemically gasified and the synthesis gas (a mixture of hydrogen and carbon oxides) bubbled through specially designed fermenters. A microorganism that is capable of converting the synthesis gas into ethanol is used under specific process conditions. The second thermochemical bioethanol production process does not use any microorganisms. In this process, biomass materials are first thermochemically gasified and the synthesis gas passed through a reactor containing catalysts, which cause the gas to be converted into ethanol (Balat et al., 2008; Caraballo, 2005).

The major biochemical conversion processes include pretreatment, hydrolysis, fermentation and distillation. During pretreatment, biomass materials undergo a physical or chemical process (thermochemical) to fractionate the lignocellulosic complex into soluble and insoluble components. Soluble components include mixtures of five- and six-carbon sugars and some oligomers. Insoluble components include cellulosic polymers, large oligomers and lignin. In hydrolysis, the pretreated material, primarily cellulose, is guided through a chemical reaction that releases the readily fermentable sugar, glucose. This step is usually accomplished with enzymes or acids. The sugars are then fermented to produce ethanol and CO_2 (Balat 2011). The biochemical approach has been attractive background and the target of several studies (Detroy et al., 1981; Hahn-Hägerdal et al., 2006; Martin and Thomsen, 2007; Saha et al., 2004; Tayeh et al., 2014; van Zyl et al., 1988).

1.3 Feedstock: lignocellulosic biomass

Lignocellulosic biomass has been considered the most attractive feedstock for ethanol production, since lignocellulose it is the most abundant biopolymer on Earth and very rich in saccharides (Lin and Tanaka, 2006; Sanchez and Cardona, 2008). These materials are renewable, abundantly available and widely distributed, been projected to be one of the main resources for cost-competitive and sustainable bioethanol production (Limayem and Ricke, 2012; Sanchez and Cardona, 2008; Sarkar et al., 2012). Biomass is unique among renewable energy sources in that it can be easily stored until needed and provides a liquid fuel alternative for use in today’s transportation system, besides their potential use for the production of others bio-based products (Demirbas, 2009; FitzPatrick et al., 2010; Ghatak, 2011).

In fact, lignocellulosic materials represent a promising option as a feedstock for bioethanol production. These materials can be classified in groups based on type of resource: agricultural and forestry biomass, dedicated energy crops, agro-industrial residues and municipal solid wastes. Lignocellulosic biomass is composed mainly of plant cell walls, with the structural carbohydrates, cellulose and hemicelluloses and heterogeneous phenolic polymer lignin as its primary components (Sjöström, 1993). However, their proportions vary substantially, depending on the type, the species, and the source of the biomass (Saini, et al., 2013; Sjöström, 1993).

Cellulose, the main constituent of the plant cell wall, is a polysaccharide composed of linear glucan chains linked together by β-1,4-glycosidic bonds with cellobiose residues as the repeating unit at different degrees of polymerization. The cellulose chains are packed into microfibrils which are stabilized by hydrogen bonds. These fibrils are attached to each other by hemicelluloses and amorphous polymers of different sugars as well as other polymers such as pectin and covered by lignin. The cellulose microfibrils which are present in the hemicellulose-lignin matrix are often associated in the form of bundles or macrofibrils. Hydrogen bonding within a cellulose microfibril determines ‘straightness’ of the chain but inter-chain hydrogen bonds might introduce order (crystalline) or disorder (amorphous) into the structure of the cellulose (Arantes and Saddler, 2010; Wyman et al., 2004; Yang et al., 2011).

Hemicelluloses are the second most abundant polymer and differ from cellulose in that they are not chemically homogeneous. Hemicelluloses are branched, heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolysed. Hemicelluloses differ in composition, in agricultural biomass, like straws and grasses are composed mainly of xylan, while softwood hemicelluloses contain mainly glucomannan. In many plants, xylans are heteropolysaccharides with backbone chains of 1,4-linked β-D-xylopyranose units. In addition to xylose, xylan may contain arabinose, glucuronic acid, or its 4-O-methyl ether, acetic acid, ferulic and *p*-coumaric acids. Hemicelluloses are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network. Hemicelluloses are also covalently attached to lignin, forming together with cellulose to form a highly complex structure (Gírio et al., 2010; Wyman et al., 2004).

Lignin is a very complex molecule constructed of phenylpropane units linked in a three dimensional structure which is particularly difficult to biodegrade. It is the most abundant aromatic heterogeneous polymer formed by phenolic compounds and their precursors are three aromatic alcohols namely, (1) *p*-coumaryl, (2) coniferyl and (3) sinapyl alcohols, which are bonded together with over two-third being ether bonds (C–O–C) and the rest being C–C bonds. The respective aromatic constituents in the polymer are called phydroxyphenyl (H), guaiacyl (G) and syringyl (S), the structure of lignin. Lignin is the most recalcitrant component of the plant cell wall, and the higher the proportion of lignin, the higher the resistance to chemical and enzymatic degradation. Generally, softwoods contain more lignin than hardwoods and most of the agriculture residues. There are chemical bonds between lignin and hemicellulose and even cellulose. Lignin is one of the drawbacks of using lignocellulosic materials in fermentation, as it makes lignocellulose resistant to chemical and biological degradation (Rowell, 2013; Sjöström, 1993).

Fig 1.1 shows the lignocellulosic material structure. In general plant cell walls are subdivided as primary (PW) and secondary (SW) walls. The distribution of cellulose, hemicellulose and lignin varies considerably among these layers. The secondary wall is composed of SW1, SW2 and SW3 where SW2 is usually thicker than the others and contains the major portion of cellulose. The middle lamella, which binds the adjacent cells, is almost entirely composed of lignin.

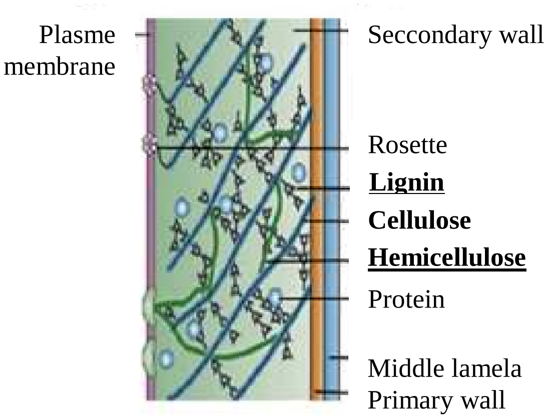

Figure 1.1 Distribution of cellulose, hemicellulose and lignin in the plant cell wall. Adapted from Menon and Rao, 2012.

The bioethanol yield from biomass, is directly related to cellulose, hemicellulose, and individual sugar concentration in the feedstock (Kim and Dale, 2004). The composition of some lignocellulosic feedstocks with potencial use in 2G bioethanol production is shown in the Table 1.1.

Among the lignocellulosic feedstocks employed for 2G bioethanol production, agricultural and forestry residues have been highlighted, due the quantitative availability comparing the others lignocellulosic biomass (Perlack, et al., 2005; Saini et al., 2014). In addition, application of residues in bioprocesses instead of the dedicated crops is advantageous because it has low cost and not only provides alternative substrates but also helps to solve their disposal problem.

Table 1.1 Composition of several lignocellulosic materials.

Feedstock	(% dry weight)		
	Cellulose	Hemicellulose	Lignin
Barley straw	34.3	23	13.3
Corn cob	36.75	29.98	23.13
Corn stover	37	31.3	17.8
Corn straw	42.1	32.6	17.5
Eucalyptus thinnings	42.2-45.8	23.8-25.5	24.36-27
Olive stone	28.1-40.4	18.8-32.2	25.3-27.2
Olive tree pruning	26.1-36.6	20-25	17.9-27.7
Pine	32.09	14.22	31.15
Rapessed straw	36.59	27.76	1.58
Rice husk	42.2	18.47	19.4
Rice straw	32-47	19-27	5-24
Sorghum bagasse	42.6	25.2	13.4
Sugarcane bagasse	47.5	20	30
Wheat bran	26	33	4
Wheat straw	33-40	20-25	15-20

Banerjee et al., 2009; Cotana et al., 2014; Díaz et al., 2010; Irbe et al., 2014; McIntosh et al., 2012; Moniz et al., 2013; Ortiz and Oliveira Jr., 2014; Ranjan and Moholkar, 2013; Romero-García et al., 2014; Sánchez et al., 2013; Saha and Cotta, 2010; Saha et al., 2013; Saini et al., 2013; Talebnia et al., 2010.

1.4 Second-generation bioethanol production in Southern Europe: Identifying lignocellulosic materials for use as feedstock

Countries geographically considered part of Southern Europe are divided in three regions:

- Southwestern Europe (Iberian peninsula): Andorra, Gibraltar, Portugal and Spain
- Southcentral Europe (Italian Peninsula): Italy, San Marino and Vatican City
- Southeastern Europe (Balkan Peninsula): Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Kosovo, Macedonia, Montenegro, Romania, Serbia, Slovenia and Turkey
- Island countries: Cyprus and Malta.

This region has a great potential in the 2G bioethanol production (Faraco and Hadar, 2011). Wheat straw is one of the feedstock with major potential use in the industrial production for 2G bioethanol worldwide and is widely available in the Southern Europe (Talebnia et al., 2010). In addition of this feedstock, eucalyptus residues and olive tree pruning also available in this region have been the aim of several studies to 2G bioethanol production (Cara et al., 2008; McIntosh et al., 2012; Negro et al., 2014). The chemical composition and availability of these residues in the Southern Europe have turned these feedstocks very attractive for this purpose. Furthermore the complementary seasonality between eucalyptus residues, wheat straw and olive tree pruning is other fact that become attractive the use of these three feedstocks in 2G bioethanol production. ER is available throughout the year, WS is available in late spring and early summer and PO is available in winter and early spring, which further potentiates their use in a multi-feedstock biorefinery under continuous operation throughout the year. Moreover, these materials have a low commercial value. For all those reasons the use of eucalyptus residues, wheat straw and olive tree pruning for 2G bioethanol production can be a sustainable alternative in the Southern Europe in replacement to fossil fuels.

1.4.1 Eucalyptus residues

Eucalyptus species are the main wood source for the production of pulp and paper in Southern Europe. It is usually managed in short rotations (10-12-year long). In the pulp-value chain, only 50-60% of the woody biomass is utilized in the pulp process (Rockwood et al., 2008). The remaining lignocellulosic material, including branches and leaves, together with bark and wood shaving rejects, are considered wastes or by-products of this industry.

The chemical composition of eucalyptus residues could slightly differ depending on the specie, harvesting period and to the heterogeneity of feedstock where the different proportions of branches, leaves and bark are present. Cellulose, hemicellulose and lignin content in eucalyptus residues was observed in the range 36-40; 18-24; 20-26% (w/w), respectively (Canettieri et al., 2007; McIntosh et al., 2012).

Eucalyptus residues can be considered an attractive, non-seasonable and inexpensive renewable raw material for 2G bioethanol production. Its chemical composition with the potential to obtain sugars suggests the biochemical route as an interesting approach. Usually the eucalyptus residues can be have been used to produce bioenergy used in the pulping process (Gavrilescu, 2008).

According to Instituto Nacional de Estatística/Instituto Florestal Nacional - INE/IFN (2013), in Portugal the eucalyptus (*Eucalyptus globulus* dominated by species) is the main occupation of the continent in forest area (812,000 ha). The annual eucalyptus productivity in Portugal is approx. 12 ton/ha and in accordance with Campilho (2006), the availability of eucalyptus residues in Portugal is estimated in $1.1\text{x}10^6$ ton. The Estratégia Florestal Nacional (ICNF, 2006) proposes the enhancement of forest biomass including innovative approaches to production of biomass/biofuels).

1.4.2 Wheat straw

An important crop grown in the Southern Europe is wheat. It is an annual, rain-fed crop cultivated predominantly in winter, planted in autumn and harvested in later spring/early summer. This crop have a potential of generate a very large amount of residues, it is estimated that the straw production ratio is 1.3 ton per ton of wheat grains (Kim and Dale, 2004).

The overall chemical composition of wheat straw can slightly differ depending on wheat species, soil, and climate conditions. Cellulose, hemicellulose and lignin content of wheat straw are in the range of 33–40, 20–25, and 15–20% (w/w), respectively (Talebnia et al., 2010).

Wheat straw is a residue that presents many interesting characteristics that facilitate its upgrade for 2G bioethanol production. It is rich in polysaccharides, is an herbaceous crop of soft material that can be transported in relatively high density form and typically has a low water content that enables its easy storage. In many countries, wheat straw is considered to be the crop residue that presents the highest potential for the production of 2G bioethanol (Talebnia et al., 2010). This residue has been used mostly for low value applications such as mulch (Bilalis et al., 2003), animal-feed (Kumar and Gomes, 2008), bedding (Ward et al., 2000) and also for energy and pulp production (Thomsen et al., 2006).

In accordance with Direção Geral de Alimentação e Veterinária - DGAV (2014), in Portugal is cultivated the durum wheat *(Triticum durum Desf.)*, common wheat *(Triticum aestivum L.)* and hybrid *(Triticosecale Wittm. ex A. Camus)*. According to Instituto Nacional de Estatística - INE (2013), in 2012 it was cultivated 54,593 ha of wheat in the country, being produced 58,990 ton of this cereal, with a potential to generate more than 7.6×10^4 ton of straw.

1.4.3 Olive tree pruning

Olive tree is a characteristic crop in Mediterranean climate. Olive groves are found throughout whole this region, where they are one of the most important agricultural waste crop. Almost all the 9.98 million hectares of olive tree cultured in the worldwide surface are grown in the Mediterranean Sea basin (Romero-García et al., 2014). Such crops require pruning in interval of 1 or 2 years, having a potential to generate a large amount of residues. As a rule, the pruning must be performed, when the vegetative activity of the olive is minimum, i.e. winter and early spring. According Romero-García et al., (2014), this procedure can generate the equivalent to approx. 1.5 ton biomass (olive tree pruning) per hectare. These residues are constituted by leaves and thin and thick branches.

Cellulose, hemicellulose, lignin content and extractive content from OP biomass are in the range of 26.1–36.6; 20.0–25.0; 17.9–27.7 and 14.1–31.4% (w/w), respectively (Romero-García et al., 2014).

So far, the management of pruning residue has generally represented a disposal problem, rather than an opportunity for additional revenue. Pruning residue is either mulched or piled and burned, at a cost estimated to about 100 and 200€ per ha, respectively (Spinelli and Picchi, 2010). Nonetheless have been proposed the use of olive tree pruning to 2G bioethanol production, not only due to the availability and its rich polysaccharide composition, but also due to its special feature of presenting high glucose content in the extractives resulted from non-structural glucans present in olive leaves (Romero-García et al., 2010).

The olive is a crop with great traditions and decisive importance in vast areas of Portugal. Its culture has a significant economic and social relevance in many regions and populations. The culture of the olive grove is distributed by all agricultural regions of the continent, being the Galician variety the most representative. According to Estatísticas Agrícolas - EA (2011), 335.841 ha of olive trees are distributed by the country, having a potential to generate more than 5.0×10^5 tons of olive tree pruning.

1.5 Steps to 2G bioethanol production by biochemical approach

Biochemical conversion of lignocellulosic materials through saccharification and fermentation is a major pathway for bioethanol production from biomass. The basic process steps in bioethanol production from lignocellulosic materials are: pretreatment, hydrolysis, fermentation and product upgrading and recovery (Balat, 2011).

During pretreatment, the biomass undergoes a physic, chemical and/or biological process to reduce the connections between the components of the lignocellulosic structure, resulting in the majority of cases in the hydrolysis of hemicellulose (Carvalheiro, et al., 2008). This process allows the improvement of biomass deconstruction during the posterior hydrolysis step (Gharpuray et al., 1983; Grethlein, 1984; Wyman, et al., 2004, Zakaria et al., 2014). A hydrolysate conditioning and/or neutralization process can be used to adjust the pH of and purify the pretreated material. In hydrolysis, the pretreated material, primarily cellulose, is guided through a chemical reaction that releases the readily fermentable sugar, glucose (Wyman et al., 2004). This step is usually accomplished with enzymes although can be used acids (strong and dilute) (Goldstein, 1983; Kassim and El-Shahed, 1986; Yang et al., 2011). In fermentation, the most common approach is to introduce a fermenting microorganism into hydrolysates sugar-rich. The purpose of the final step, product upgrading and recovery, is to separate the fuel from the water and residual solids, to reach >99% purity for bioethanol, which could involve mainly distillation and dehydration (Balat, 2011).

Fig. 1.2 shows the schematic of the production of 2G bioethanol by biochemical approach.

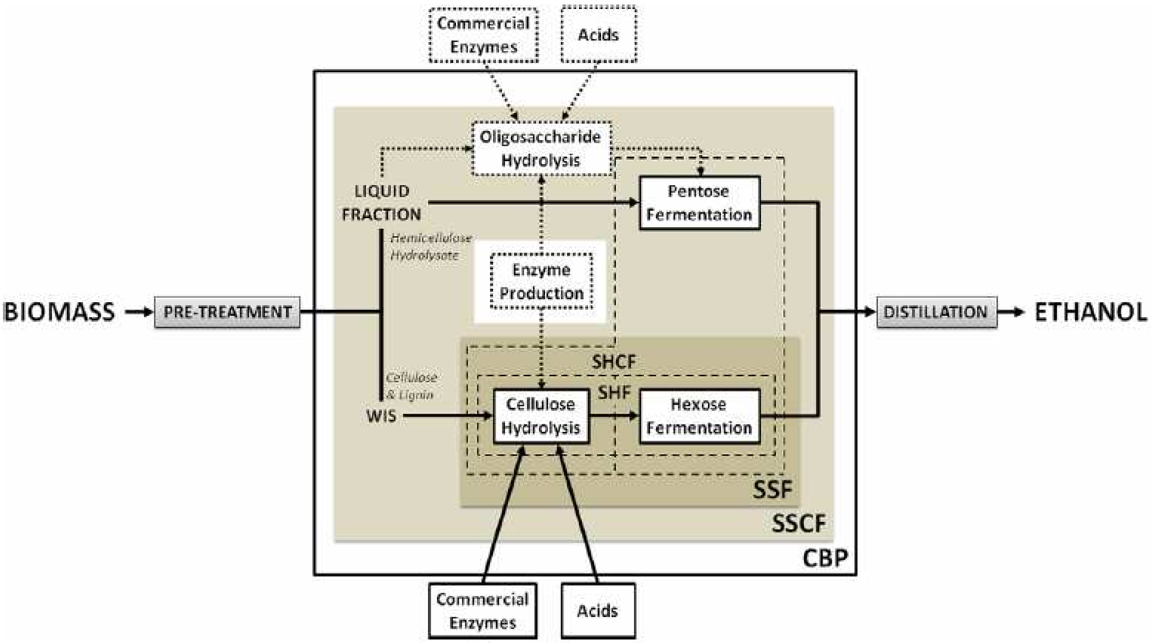

Figure 1.2 Schematic representation of process steps and its integration for 2G bioethanol production by biochemical routes (Gírio et al., 2010).

Although the 2G technology is very attractive, is not well established yet, as some of its processes require significant improvements, including the efficient deconstruction of biomass (pretreatment and hydrolysis) to maximize the release of fermentable sugars and the effective fermentation of pentoses to maximize fermentation process efficiency, i.e. to maximize ethanol yield from total carbohydrates, improving the economics of the process (Alvira et al., 2010; Hahn-Hägerdal et al., 2006; Koppram et al., 2014).

1.6 Deconstruction of lignocellulosic biomass

The recalcitrance of lignocellulose is one of the major barriers to the economical production of bioethanol. The technical approach to overcome recalcitrance has been pretreatment of lignocellulosic biomass to remove the rigid structure composed by cellulose, hemicellulose and lignin, and make cellulose more accessible to hydrolytic enzymes towards its conversion to glucose (Alvira et al., 2010). If the pretreatment is not efficient enough the resultant residue is not easily hydrolysed by cellulolytic enzymes and if it is too severe, results in the production of toxic compounds which inhibit microbial metabolism (Alvira et al., 2010; Banerjee et al., 2010; Mood et al., 2013). The quantity and composition of the sugars stream released depend on the feedstock used and the pretreatment technology employed.

1.6.1 Pretreatment of lignocellulosic biomass

One the main processing challenge in the bioethanol production from lignocellulosic biomass is the feedstock pretreatment. Studies have shown that pretreatment is the most significant determinant of success of the cellulosic bioethanol technology because it defines the extent to and cost at which the carbohydrates of cellulose and hemicellulose can be converted to bioethanol (Alvira et al., 2010; Banerjee et al., 2010; Kumar et al., 2009). The lignocellulosic material is made up of a matrix of cellulose and lignin bound by hemicellulose chains. During the pretreatment, this matrix should be broken in order to reduce the crystallinity degree of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack beyond of increase the porosity of the materials (Alvira et al., 2010; Wyman et al., 2004).

Goals of an effective pretreatment process are: formation of sugars directly or subsequently by hydrolysis; to avoid loss and/ or degradation of sugars formed; to limit formation of inhibitory products; to reduce energy demands and to minimize costs (Alvira et al., 2010; Banerjee, et al., 2010; Kumar et al., 2009).

For bioethanol production, hemicelluloses are commonly removed during the initial stage of biomass processing aiming to reduce structural constraints for further enzymatic cellulose hydrolysis (Alvira et al., 2010). The main process options for the selective fractionation of hemicelluloses from biomass include the use of acids, water (liquid or steam), organic solvents and alkaline agents (Hendriks et al., 2009; Kumar et al., 2009; Mood et al., 2013). The latter two are not selective towards hemicellulose as they also remove lignin. Acid/water/steam pretreatments are the most commonly applied technologies yielding a selective solubilisation of hemicelluloses and producing hemicellulose-rich liquids totally or partially hydrolysed to oligomeric and monomeric sugars and cellulose-enriched solids for further bioprocessing (Carvalheiro et al., 2008). Thus, only the acid pretreatment (concentrated or dilute) and hydrothermal pretreatment (autohydrolysis and steam explosion) will be presented in more detail. Others pretreatments are summarized in the Table 1.2 and shortly presented with their main advantages and disadvantages.

Acid treatment

Generally, acid pretreatments employ the use of sulfuric acid although nitric, phosphoric or hydrochloric acids are also employed to remove hemicellulose components and expose cellulose for enzymatic digestion (Carvalheiro et al., 2008). This pretreatment method gives high reaction rates and significantly improves cellulose hydrolysis. The acid pretreatment can operate either under a low temperature and high acid concentration (concentrated acid pretreatment) or under a high temperature and low acid concentration (dilute acid pretreatment).

Concentrated acids, beyond the hydrolysis of the hemicellulosic fraction, are powerful agents for cellulose hydrolysis. The acid concentration used in concentrated acid hydrolysis process is in the range of 10–30%. Reaction times are typically of 10-12h, which are much longer than for dilute acid process due to the lower temperature employed (Hamelinck et al., 2005). Concentrated acid-based processes are very expensive and cause significant operational problems. Acid recovery is a key step for economic viability of concentrated acid pretreatment (Goldstein et al., 1983).

Hydrolysis with dilute acid has been successfully developed given that high reaction rates can be achieved, also improving significantly the subsequent process of cellulose hydrolysis. Usually, diluted acids are added to biomass at concentrations up to 4% (w/w), followed by constant mixing at temperatures between 130 and 210°C (Menon and Rao, 2012). Depending on the conditions of the pretreatment, the hydrolysis of the sugars could take from a few minutes to hours.

There are primarily two types of dilute acid pretreatment processes: low solids loading, 5–10% (w/w), high temperature (T>160°C), continuous-flow processes; and, high solids loading, 10–40% (w/w), lower temperature (T<160°C), batch processes. In general, higher pretreatment temperatures and shorter reactor residence times result in higher soluble xylose recovery yields and enzymatic cellulose digestibility. Depending on the substrate and the conditions used, between 80% and 95% of the hemicellulosic sugars can be recovered by dilute acid pretreatment from the lignocellulosic material (Balat, 2011).

Although the dilute acid hydrolysis is usually applied to liberate the hemicellulosic sugars, it can also be used to cellulose hydrolysis. For that, two stages are necessary: the first stage is performed at low temperature to maximize the yield of hemicellulose hydrolysis; and the second stage, at higher temperature, to optimized the hydrolysis of the cellulose portion of the feedstock (Balat et al., 2011).

While acid hydrolysis is an efficient process for the hydrolysis of hemicelluloses and recovery of xylose and other hemicellulosic sugars, it alters lignin structure, presents high cost, generates corrosion of the equipment and forms toxic compounds for the fermentation process (Kumar et al., 2009). As consequence of this method, acidic pre-hydrolysates must be neutralized before the sugars proceed to fermentation.

Steam explosion

In the steam explosion method, lignocellulosic biomass is treated with high-pressure saturated steam, and then pressure is swiftly reduced, which makes the materials undergo an explosive decomposition. This method has been described as a thermomechanochemical process where the breakdown of structural components is aided by heat in the form of steam (thermo), shear forces due to the expansion of moisture (mechano), and hydrolysis of glycosidic bonds (chemical) once it is (self)-catalysed (e.g., by the biomass derived acetic acid, and possibly by added catalysts) (Carvalheiro et al., 2008; Gírio et al., 2010). More specifically, biomass particles are heated using pressurized steam (20–50 bar, 160-270ºC) for several seconds to a few minutes and then the pressure is released to atmospheric pressure, condensed moisture evaporates and desagregation of lignocellulosic matrix takes place (Mood et al., 2013). The forces resulting from decompression lead to a desagregation of lignocellulosic matrix, breaking down inter- and intra-molecular linkages. Uncatalyzed steam explosion refers to a pretreatment technique in which lignocellulosic biomass is rapidly heated by high pressure steam without addition of any chemicals (Balat, 2011). Most steam treatments yield high hemicellulose solubility (producing mainly oligosaccharides) along with slight lignin solubilisation (Gírio et al., 2010). However, the use of saturated steam at too high pressure causes hemicellulose degradation and lignin transformation (Kumam et al., 2010). To summarize, the effects of steam explosion treatment on lignocellulosics are: (1) increases crystallinity of cellulose by promoting crystallization of the amorphous portions; (2) hemicellulose is easily hydrolyzed; (3) there is evidence that steam explosion promotes delignification.

Studies without added catalyst report sugars recoveries between 45% and 69% (Gírio et al., 2010). Addition of H_2SO_4 (or SO_2) or CO_2 (typically 0.3–3% (w/w)) in steam explosion can decrease time and temperature, effectively improve hydrolysis, reduces the formation of sugar degradation products, and lead to complete removal of hemicellulose increasing maximum pentose yield (Gírio et al., 2010). SO_2 appears more appealing than H_2SO_4 in steam explosion since the former requires much less expensive reactors, generates less gypsum, yields more xylose, and produces more digestible substrate with high fermentability. The main drawback of SO_2 is its high toxicity, which may pose safety and health risks. However, SO_2 is used in various industrial processes using well-established techniques (Balat, 2011).

Due to its lower capital investment and higher energy efficiency, steam explosion is among the very limited number of cost-effective pretreatment technologies for pilot scale demonstration and commercialized applications (Mood et al., 2013).

Liquid hot water

One of the most promising methods for lignocellulosic biomass pretreatment is the liquid hot water process, which uses compressed liquid hot water (pressure above saturation point) (Gírio et al., 2010). The processes with liquid water under high temperature and pressure are also called hydrothermal treatment, hot compressed water (HCW), hydrothermolysis, aquasolve process, aqueous processing and pressure-cooking in water (Ruiz et al., 2013). The operation temperatures usually range between 150 and 230°C and the reaction time may vary from seconds up to hours, depending on the temperature. Solids concentration, usually referred as the liquid-to-solid ratio (LSR), may range between 2 and 100 (w/w), although the most common values are around 10 (Gírio et al., 2010).

In this autohydrolysis process, where water is the only reactive added to substrate, catalysts of hydrolysis are in a first stage hydronium ions (H_3O^+) coming from water auto-ionization that lead to depolymerization of hemicelluloses by selective hydrolysis of both glycosidic linkages and acetyl groups. In a second stage, hydronium ions coming from acetic acid also act as catalysts, improving reaction kinetics. The contribution of hydronium ions from acetic acid is higher than that from water autoionization. Hydrolysis of uronic acids should also occur in first stages of treatment, simultaneously with acetyl groups. Despite of their resistance to hydrolysis, uronic acids may also contribute to formation of hydronium ions, but their role in hydrolysis is still not completely understood. In this process, acids resulting from hydrolysis of acetyl and uronic groups, originally present in hemicelluloses, catalyze hydrolysis of links between hemicelluloses and lignin as well as hydrolysis of carbohydrates (Carvalheiro et al., 2008).

This pretreatment has been reported to have the potential to enhance cellulose digestibility, sugar extraction, and pentose recovery, with the advantage of producing pre-hydrolysates containing little or no inhibitory effect on sugar fermentation. It reduces the need for neutralization of liquid streams and conditioning chemicals since acid is not added (Menon and Rao, 2012). The mild pH and lower corrosion of equipments are benefits of the authohydrolysis (Carvalheiro et al., 2008). The main drawback of this process when using the liquid stream for fuel ethanol fermentation is that the solubilized hemicellulose appears mainly in oligomeric form (Gírio et al., 2010).

Operating conditions of hydrothermal treatment

The most important operational variables of hydrothermal processing include temperature, residence time, particle size, moisture content (ratio liquid/solid) and pH (Ruiz et al., 2013). The relationship between temperature and time strongly influences the physical–chemical characteristics of lignocellulosic materials in hydrothermal processing. The hemicellulose-sugars degradation is observed at higher temperatures and residence times, i.e. more severe operational conditions lead to losses of hemicellulosic sugars (Carvalheiro et al., 2009). For this reason, a strict control is required for high temperature reactions due to thermal degradation. Several works showed that the products (pentose yield) from hydrothermal processing are favored at lower reaction temperatures and longer residence times. Normally, when larger particle sizes are used, heat transfer problems lead to over cooking of the exterior (with consequent formation of inhibitors) and incomplete hydrolysis of the interior. This problem can be overcome by reducing particle size as the first pretreatment step. This size reduction process not only changes the particle size and shape, but also increases bulk density, improves flow properties, increases porosity, increases surface area and is usually required to make material handling easier before hydrothermal processing. The higher surface area increases the number of contact points for chemical reaction. However, the utilization of very small particles in hydrothermal processing would not be desirable to optimize the effectiveness of the process and improve economy, due to the significant energy requirements of particle reduction process (Ballesteros et al., 2000). According to Ruiz et al., (2013), the use of blends with different particle size distributions has a selective influence over the sugar extraction: thus, the use of a blend with defined percentages of the various particle sizes is recommended before carrying out a hydrothermal processing. Moisture content and ratio liquid/solid may also greatly influence the ability of heat and chemicals (H_3O^+) to penetrate lignocellulosic materials, causing an uneven treatment of material. An uneven treatment can potentially result in the selective degradation of the outer portion of the lignocellulosic materials, while at the same time the interior is less affected by the treatment. The moisture content has a dramatic effect on the efficacy of the hydrothermal processing as a substantial decrease in the amount of hemicellulose-derived carbohydrates recovered in the water-soluble fraction (Cutis et al., 2004). It is possible to obtain high glucose, xylose, arabinose and acetic acid concentrations by combining high temperatures with a medium-low treatment time and liquid/solid ratio (Rodrigues et al., 2009). On the other hand, the formation of hydronium ions from water and from organic acids is an important factor during hydrothermal processing, since the lignocellulosic materials and water mixture will reach high temperatures and pressures during the process. These high temperatures and pressures will accelerate the acid-catalyzed hydrolysis of cellulose and hemicellulose as well as the acid-catalyzed degradation of glucose and xylose. Monitoring and control of the pH in hydrothermal processing will maximize the solubilization of the hemicellulose fraction as liquid soluble oligosaccharides while minimizing hydronium ions concentration and, more importantly, the degradation of these oligosaccharides and monosaccharides to degradation products (Ruiz et al., 2013).

Effect of hydrothermal treatment on cellulose

Hydrothermal processing alters the structure of cellulose promoting the enzyme accessibility in the pretreated material increasing the potential of cellulose saccharification. Due to solubilization of the hemicellulose and/or lignin, physical changes that improve enzymatic saccharification include an increase in pore size to enhance enzyme penetration and an increase in accessible area that has been shown to correlate well with the susceptibility of these substrates to enzyme saccharification using hydrothermal processing as pretreatment. Degradation of the cellulose after hydrothermal processing can be observed at different temperatures (>230°C) (Ruiz et al., 2013).

Table 1.2 Advantages, limitations and disadvantages of several pretreatment processes for lignocellulosic materials.

Pretreatment process	Advantages	Limitations and disadvantages
Acid hydrolysis	- Hemicellulose is hydrolysed to xylose and other sugars and alters lignin structure	- High cost; equipment corrosion and formation of toxic substances
AFEX[b]	- Increases accessible surface area; removes lignin and hemicellulose to an extent and does not produce inhibitors for down-stream processes	- Not efficient for biomass with high lignin content
Alkaline hydrolysis	- Removes hemicelluloses and lignin and increases accessible surface area	- Long residence times required and irrecoverable salts formed and incorporated into biomass
Biological	- Simple equipment; degrades lignin and hemicelluloses and low energy requirements	- Rate of hydrolysis is very low
CO_2 explosion	- Increases accessible surface area; cost-effective and does not cause formation of inhibitory compounds	- Does not modify lignin or hemicelluloses
Ionic liquids	- Remove hemicellulose and lignin; generate amorphous and porous cellulose	- Still in initial stages
LHW[a]	- Removal of hemicellulose; making enzymes accessible to cellulose	- Long residence time and less lignin removal
Mechanical	- Reduces cellulose crystallinity	- Power consumption is higher than inherent biomass energy
Organosolv	- Hydrolyzes lignin and hemicelluloses	- Solvents need to be recovered and high cost
Ozonolysis	- Reduces lignin content and does not produce toxic residues	- Large amount of ozone required and expensive
Pulsed electrical field	- Ambient conditions and disrupts plant cells	- Process needs more research
Steam explosion	- Causes hemicellulose degradation and lignin transformation; cost-effective	- Incomplete disruption of the lignin-carbohydrate matrix and generation of compounds inhibitory to microorganisms

[a] Liquid hot water; [b] Ammonia and carbon dioxide

Alvira et al., 2010; Gírio et al., 2010; Mood et al., 2013; Hendriks and Zeeman, 2009; Kumar et al., 2009; Menon and Rao, 2012; Sanchez and Cardona, 2008.

Effect of hydrothermal treatment on hemicellulose

Hydrothermal processing is a suitable method for hemicellulose depolymerization, under selected operational conditions as hemicellulose can be almost totally removed from lignocellulosic materials, being decomposed into valuable soluble products such as oligosaccharides, monosaccharides, sugar-degradation products (such as furfural or 5-hydroxymethylfurfural (HMF)) and acetic acid (from acetyl groups hydrolysis). Furthermore, when xylan is subjected to hydrothermal processing under mild temperature, high-molar mass xylooligosaccharides (XOS) and xylose are produced, being the major products derived from hemicellulose present in the liquor phase (Carvalheiro et al., 2008; Ruiz et al., 2013).

Effect of hydrothermal treatment on lignin

During the hydrothermal processing lignin and lignin–hemicellulose linkages can undergo degradation, partial depolymerization and profound re-localization. Moreover, the fraction of solubilized lignin depends on the operation conditions (severity of reactions conditions) and on the raw material. Two-phase mechanism can be suggested for lignin reaction: (1) a very fast reaction where lignin fragments with low molecular weight and high reactivity are solubilized by breaking lignin–carbohydrate bonds into soluble fragments; (2) as lower reaction where the soluble fragments react with one another by re-condensation and lignin re-polimerization, which also occurs in the presence of the organic acids liberated in the hydrothermal processing. The partial de-polymerization of lignin and breaking of lignin–hemicellulose linkages produced part of the phenolics present in the hydrothermal processing liquors (Ruiz et al., 2013).

1.7 Toxicity of hemicellulosic hydrolysates

Depending on the process and conditions used during pretreatment, a great amount of compounds that can seriously inhibit the subsequent fermentation are formed in addition to fermentable sugars, decreasing the yields and productivities of bioethanol (Almeida et al., 2007; Delgenes et al., 1996; Palmqvist and Hahn-Hägerdal, 2000a; 2000b). Several pretreatment processes generate toxic compounds and inhibitors of enzymes and fermentation microorganisms, resulting from their severe conditions. Inhibitors derived from pretreatment include sugar degradation products, furan derivatives, organic acids and phenolic compounds. These compounds are principally generated from lignocellulosic components during pretreatment. Sugar degradation products, including furfural and HMF, are derived from the degradation of hemicellulose and cellulose sugars. Under high temperature and pressure, sugar monomers (hexose), namely, mannose, galactose and glucose, are degraded to HMF, whereas xylose and arabinose are degraded to furfural. Furthermore, formic acid is formed when furfural and HMF are broken down, and levulinic acid is formed by HMF degradation (Palmqvist and Hahn-Hägerdal, 2000b). In addition, acetic acid is released by the deacetylation of hemicelluloses and lignin. The breakdown of lignin produces hydroxybenzaldehyde, vanillin, the degraded product of guaiacylpropane units and soluble lignin (Palmqvist and Hahn-Hägerdal, 2000b). Furthermore metal ions can be generated as a consequence of corrosion of the equipment. Figure 1.3 shows some reactions occurring during pretreatment of lignocellulosic materials.

The sugar degradation products (furfural and HMF) promote inhibition of glycolysis particularly by interfering with dehydrogenase activities. The weak organic acids (acetic, formic and levulinic acids), can promote the inflow of liposoluble, undissociated acids into cytosol and disruption of energy generation due to disruption of pH gradient in cytosol. The phenolic derivates have different effects, vanillin loss of membrane integrity, hydroxybenzaldehyde disruption of sugar transport and soluble lignin promote deactivation of cellulase enzymes (enzyme precipitation)/non-productive enzyme adsorption (Almeida et al., 2007; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hägerdal, 2000b; Pienkos et al., 2009).

For these reason presented, depending on the type of pretreatment employed and the different tolerances of the fermenting microorganisms to the inhibitors, detoxification can be employed as a methodology to remove or reduce inhibitors content of the hemicellulosic hydrolysates.

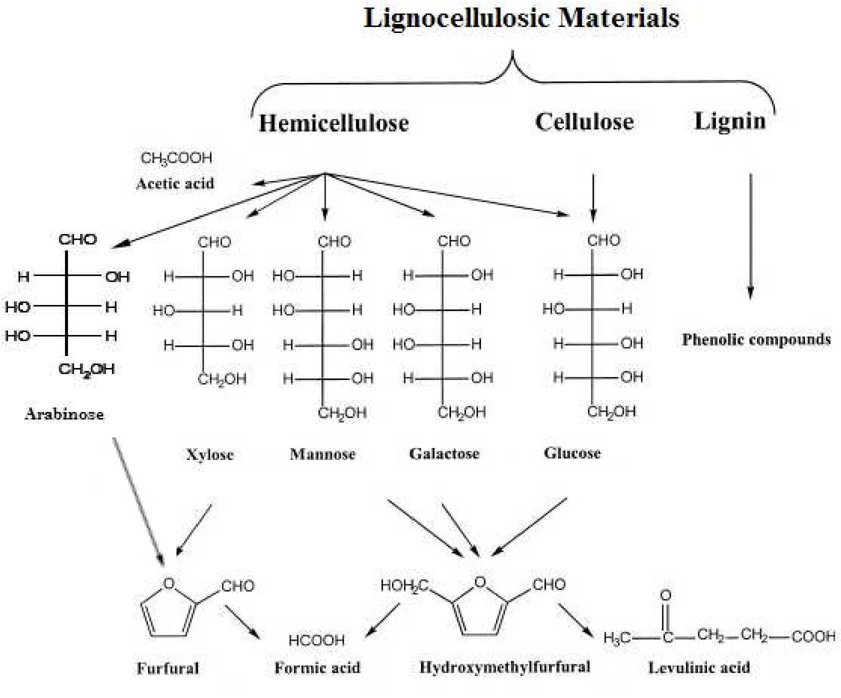

Figure 1.3 Reactions occurring during pretreatment of lignocellulosic materials. Adapted from Palmqvist and Hahn-Hägerdal, 2000b.

A number of detoxification methods including biological, physical, and chemical have been proposed to transform inhibitors into inactive compounds or to reduce their concentration. The more used are evaporation, overliming, adsoption in activated charcoal, diatomaceous earth and ion exchange resins, and use of ligninolytic enzyme laccase, pre-fermentation with the filamentous fungus *Trichoderma reesei* (Mussatto and Roberto, 2004).

1.8 Enzymatic hydrolysis of lignocellulosic biomass

Enzymatic hydrolysis of cellulose is carried out by cellulolytic enzymes. These enzymes are produced by several microorganisms, commonly by bacteria and fungi (Yang et al., 2011). The products of the hydrolysis are usually reducing sugars including glucose. Although the commercial enzymes are still of high costs, there are other advantages that make it attractive process, such as operating processes under mild conditions (pH 4.8 and temperature 45–50°C) obtaining higher yields when compared to the chemical process, and no degradation components of glucose are formed (Duff and Murray, 1996; Sun and Cheng, 2002). Cellulolytic enzymes are a group of enzymes that synergistically hydrolyze cellulose (Fig. 1.4). The widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic actions by endoglucanase (EG, endo-1,4-D-glucanohydrolase); exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase) and β-glucosidase (BGL, cellobiases) (Saha, 2004; Sun and Cheng, 2002). EG play an important role in the cellulose hydrolysis by cleaving cellulose chains randomly and thus promoting further degradation. EG hydrolyze accessible intramolecular β-1,4-glucosidic bonds of cellulose chains randomly to produce new chain ends. CBH progressively cleave cellulose chains at the ends to release soluble cellobiose. BGL complete the hydrolysis process by catalyzing the hydrolysis of cellobiose to glucose (Balat et al., 2008; Zhang and Lynd, 2004). Endoglucanases especially act on amorphous cellulose, whereas cellobiohydrolases can act on crystalline cellulose as well (Sanchez and Cardona, 2008).

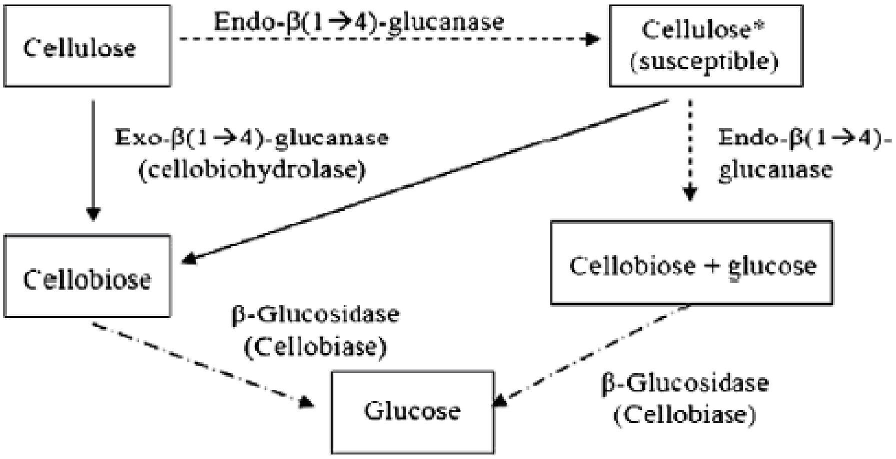

Figure 1.4 Mode of action of cellulolytic enzymes (Balat, 2011).

The enzyme β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose as both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose. Thus, β-glucosidase not only produces glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. However, like β-glucanases, most β-glucosidases are subject to end-product (glucose) inhibition (Saha, 2004). Several methods have been developed to reduce the inhibition by cellobiose and to a lesser extent by glucose, including the use of high concentrations of enzymes, the supplementation of β-glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (Modenbach, et al., 2013; Sun and Cheng 2002).

Gluco- and xylooligosaccharides also have inhibitory effects on cellulolytic enzymes (Koppram et al., 2014; Zhang et al., 2012). Although the mechanism of this inhibition is not entirely understood, it is believed that the hydrolysis of xylooligomers into less inhibitory xylose significantly improves glucose production yields (Qing et al., 2010; Zhang et al., 2012). Thus, significantly reduced enzyme dosage and cost could be achieved by enhancement of xylanase and β-xylosidase activities in the widely used cellulolitic preparations. The presence of increased xylanolytic activities in the latest cellulolytic enzyme preparations leads to improved overall saccharification yields (Koppram et al., 2014; Zhang et al., 2012).

In addition to the three major groups of cellulolytic enzymes, there are also a number of auxiliary enzymes that attack hemicellulose (Gírio et al., 2010). The hemicellulose hydrolysis involves a multi-enzyme system, including endoxylanase, exoxylanase, β-xylosidase, α-arabinofuranosidase, α-glucoronidase, acetyl xylan esterase, and ferulic acid esterase (Badal and Rodney, 1999). The endoxylanase attacks the main chains of xylans and β-xylosidase hydrolyzes xylooligosaccharides to xylose. The α-arabinofuranosidase and α-glucuronidase remove the arabinose and 4-O-methyl glucuronic acid substituents, respectively, from the xylan backbone. Hemicellulolytic esterases include acetyl esterases which hydrolyze the acetyl substitutions on xylose moieties, and feruloyl esterases which hydrolyze the ester bond between the arabinose substitutions and ferulic acid. Feruloyl esterases aid the release of hemicellulose from lignin and render the free polysaccharide product more amenable to degradation by the other hemicellulases (Howard et al., 2003). While the number of enzymes required for xylan hydrolysis is much greater than for cellulose hydrolysis, accessibility to the substrate is easier since xylan does not form tight crystalline structures (Balat, 2011). In the Table 1.3 the enzymes involved in lignocellulosic degradation are shown.

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulolytic enzymes onto the surface of cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of enzymes (Taherzadeh and Karimi, 2007). The irreversible adsorption of cellulolytic enzymes on cellulose is partially responsible for this deactivation. Addition of surfactants during hydrolysis is capable of modifying the the cellulose surface property and minimizing the irreversible binding of cellulase on cellulose (Sun and Cheng, 2002).

Enzyme recycling may increase the rates and yields of hydrolysis, reduce the net enzyme requirements and thus lower costs. As the cellulose hydrolysis proceeds, the adsorbed enzymes (endo- and exo-glucanase components) are gradually released in the reaction mixture. The β-glucosidase does not adsorb onto the substrate. These enzymes can be recovered and reused by contacting the hydrolysate with the fresh substrate. However, the amount of enzyme recovered is limited because some enzymes remain attached to the residual substrate, and some enzymes are thermally inactivated during hydrolysis (Saha, 2004).

Table 1.3 Enzymes involved in lignocellulose degradation.

Enzyme	Systematic name	Mode of action
Endo-l,4-β-glucanase	1,4-β-D-Glucan-4-glucanohydrolase	Endo-hydrolysis of 1,4-β-D-glucosidic linkages
Exo-l,4-β-glucanase	1,4-β-D-Glucan cellobiohydrolase	Hydrolysis of 1,4-β-D-glucosidic linkages releasing cellobiose
β-Glucosidase	β-D-Glucoside glucohydrolase	Hydrolyzes cellobiose and short cello-oligosaccharides to glucose
Endo-l,4-β-xylanase	1,4-β-D-Xylan xylanohydrolase	Hydrolyzes manly interior β-1,4-xylose linkages of the xylan backbone
Exoxylanase	-	Hydrolyzed linkage terminal β-1,4 (reducing end)
β-xylosidase	-	Hydrolyzed linkage terminal β-1,4 (non-reducing end)
α-L-Arabinofuranosidase	α-L-Arabinofuranoside arabinofurano-hydrolase	Hydrolyzes terminal non-reducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	α-Glucuronoside glucanohydrolase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Acetyl-ester acetylhydrolase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Carboxylic ester hydrolase	Hydrolyzes feruloylester bonds in xylan
Lignin peroxidase	-	Oxidation of benzilic alcohols, cleavage of C-C bonds, cleavage of C-O bonds
Manganese peroxidase	-	Catalytically dependent on H_2O_2 and Mn^{2+} ions
Laccase	Donor: hydrogen peroxide oxidoreductase	Oxidizes phenolic subunits of lignin

Balat, 2011; Evans et al., 1994; Kuhad et al., 1997; Saha, 2004.

During the enzymatic hydrolysis of cellulosic substrates, several factors restrict the sustained catalytic activity of the cellulolytic mixture. It has been suggested that these limitations are due to both substrate- and enzyme-related factors. The enzymatic degradation of cellulose is a complicated process that takes place at a solid–liquid phase boundary, where the enzymes are the mobile components. When cellulase enzyme systems act in vitro on insoluble cellulosic substrates, three processes occur simultaneously: (1) chemical and physical changes in the residual (not yet solubilized) solid-phase cellulose, (2) primary hydrolysis, involving the release of soluble intermediates from the surface of reacting cellulose molecules, and (3) secondary hydrolysis, involving hydrolysis of soluble intermediates to lower molecular weight intermediates, and ultimately to glucose (Balat et al., 2008).

There are different factors that affect the enzymatic hydrolysis of cellulose, namely, the crystallinity of cellulose, its accessible surface area and protection by lignin and hemicellulose, degree of cellulose polymerization, and degree of acetylation of hemicelluloses (Alvira et al., 2010). The substrates, cellulolytic activity, enzyme to substrate ratio, dosage of the active components (β-glucosidase/cellobiohydrolase/endoglucanase ratio) in the enzymatic mixture, reaction conditions (temperature, pH as well as other parameters), and a strong product inhibition also are factors that affect the enzymatic hydrolysis of cellulose (Balat, 2011; Lin and Tanaka, 2006; Sun and Cheng, 2002). Most of these factors are discussed below.

The cellulose microfibrils have both crystalline and amorphous regions, and the crystallinity is given by the relative amounts of these two regions. The major part of cellulose (around 2/3 of the total cellulose) is in the crystalline form. It was shown that cellulase readily hydrolyzes the more accessible amorphous portion of cellulose, while the enzyme is not so effective in degrading the less accessible crystalline portion. It is therefore expected that high-crystallinity cellulose will be more resistant to enzymatic hydrolysis, and it is widely accepted that decreasing the crystallinity increases the digestibility of lignocelluloses. This discussion may indicate that the crystallinity is an important factor in digestibility of lignocelluloses. However, it is not the only factor in effective enzymatic hydrolysis of these materials, due to the heterogeneous nature of celluloses and the contribution of other components such as lignin and hemicellulose (Taherzadeh and Karimi, 2008).

Several studies have shown a good correlation between the pore volume or population (accessible surface area for cellulase) and the enzymatic digestibility of lignocellulosic materials. The main reason for improvement in enzymatic hydrolysis by removing lignin and hemicellulose is related to the cellulose accessible surface area. The effect of this area may correlate with crystallinity or lignin protection or hemicellulose presentation or all of them. Therefore, many researchers have not considered the accessible surface area as an individual factor that affects the enzymatic hydrolysis. The accessible surface area in lignocellulosic material and its interaction with the enzymes can be limiting in enzymatic hydrolysis. The rate of hydrolysis is usually very high at first, and then decreases in the later stages. The accessible surface area per gram of substrate (m^2/g), sharply increases during the initial stage. However, it was shown that the cellulose surface area is not a major limiting factor for hydrolysis of pure cellulose. In other words, the slowdown of hydrolysis in the later stages is not due to a lack of associable surface area, but to the difficulty in hydrolysis of crystalline part of cellulose and probably also because of product inhibition. Therefore, one may expect a lower rate of hydrolysis after hydrolysis of the amorphous cellulose (Taherzadeh and Karimi, 2008).

Removal of hemicellulose increases the mean pore size of the substrate and therefore increases the accessibility and the probability of the cellulose to become hydrolyzed. Degree of acetylation in the hemicellulose is another important factor because lignin and acetyl groups are attached to the hemicellulose matrix and may hinder polysaccharide breakdown (Alvira et al., 2010).

The cellulose and hemicellulose are cemented together by lignin. Lignin is responsible for integrity, structural rigidity, and prevention of swelling of lignocelluloses. Thus, lignin content and distribution constitute the most recognized factor which is responsible for recalcitrance of lignocellulosic materials to enzymatic degradation by limiting the enzyme accessibility; therefore the delignification processes can improve the rate and extent of enzymatic hydrolysis. The composition and distribution of lignin might also be as important as the concentration of lignin. It was also shown that lignin still has a significant effect on enzymatic digestibility, even in cases where it no longer prevents fiber swelling. The reason for improved rate of hydrolysis by removal of lignin might be related to a better surface accessibility for enzymes by increasing the population of pores after removing of lignin (Taherzadeh and Karimi, 2008).

Substrate concentration affects the yield and initial rate of enzymatic hydrolysis of cellulose. Although high substrate concentration promotes high product concentration, can cause substrate inhibition which substantially lowers the rate of the hydrolysis. This fact is probably due to high solid loading has mass transfer limitations, increased medium viscosity and generate high concentrations of inhibitors, as well as increase product inhibition (Koppram et al., 2014; Modenbach and Nokes, 2013). The extent of substrate inhibition depends on the ratio of total substrate to total enzyme.

Other compounds have also been shown to inhibit the enzymes, including degradation products formed during the pretreatment, such as organic acids (formic and acetic), furan derivatives (furfural, HMF) and lignin derivatives (vanillic and syringic acids and syringylaldehyde). Furthermore, the end-product ethanol has also been observed to inhibit cellulases (Viikari, 2012).

Increasing the dosage of cellulolytic enzymes in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process. Cellulase dosage of 10 $FPU/g_{cellulose}$ is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) at a reasonable enzyme cost. Cellulase enzyme loadings in hydrolysis vary from 7 to 33 $FPU/g_{substrate}$, depending on the type and concentration of substrates (Sun and Cheng, 2002).

1.9 Fermentation

1.9.1 Fermentation of hexoses and pentoses

The sugars originated during the pretreatment and enzymatic hydrolysis of lignocellulosic material can be fermented by bacteria, yeast or filamentous fungi to bioethanol production (Hahn-Hägerdal et al., 2006). Microorganisms for bioethanol fermentation can best be described in terms of their performance parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are: temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Balat, 2011). Traditionally, the microorganism used for fermenting bioethanol in industrial processes is *Saccharomyces cerevisiae*, which has proved to be very robust and well suited to the fermentation of lignocellulosic hydrolysates (Balat, 2011). This yeast is one of the most effective ethanol-producing microorganisms, with high ethanol productivity and tolerance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass, ability to grow at relatively low pH, which prevents contamination by other bacteria (Matsushika et al., 2009a) and non-pathogenicity (Ostergaard et al., 2000).

The product of enzymatic hydrolysis of lignocellulosic materials can contain both six- (hexoses) and five-carbon (pentoses) sugars, if both cellulose and hemicellulose are hydrolyzed. The sugar composition of the lignocellulosic hydrolysates typically consists of glucose, xylose, arabinose, galactose and mannose, whose relative amounts depend on the lignocellulosic material.

Wild-type *S. cerevisiae* ferments glucose, the dominant sugar in all plant hydrolysates, at high rates even under anaerobic conditions. After uptake, glucose assimilation proceeds via the Embden-Meyerhof glycolytic pathway. In anaerobic, fermentative cultures of *S. cerevisiae*, the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is re-oxidized via alcoholic fermentation. Mannose, an isomer of glucose that occur in plant-derived biomass hydrolysates also can be fermented by all wild-type *S. cerevisiae* strains. In general, yeast strains capable of fermenting glucose can also ferment mannose. Furthermore, galactose, another sugar can be fermented by *S. cerevisiae*, this sugar being converted into glucose-6-phosphate via the Leloir pathway as shown in the Fig. 1.5 (Madhavan et al., 2012).

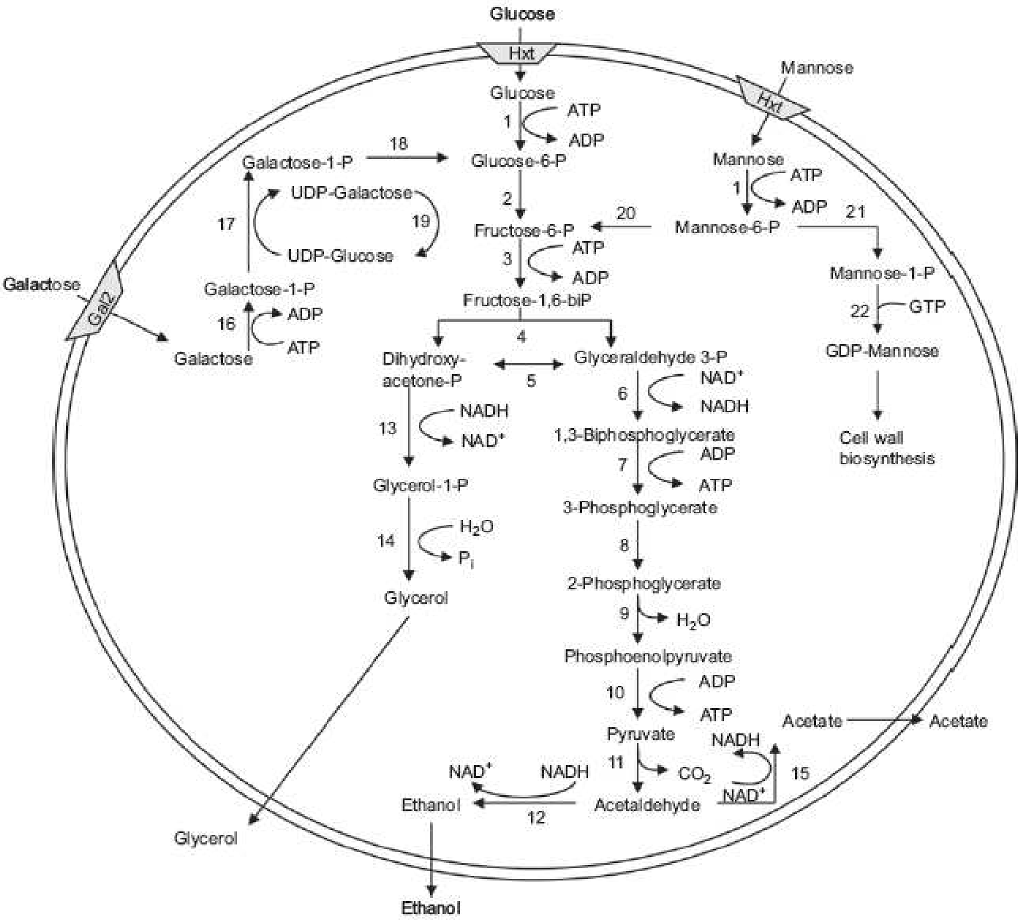

Figure 1.5 Metabolic routes for assimilation of the hexose sugars glucose, galactose and mannose by the Embden-Meyerhof-Parnas pathway. 1, Hexokinase; 2, Phosphoglucose isomerase; 3, Phosphofructokinase; 4, Aldolase; 5, Triosephosphate isomerase; 6, Glyceraldehyde-3-phosphate dehydrogenase; 7, Phosphoglycerate kinase; 8, Phosphoglycerate mutase; 9, Enolase; 10, Pyruvate kinase; 11, Pyruvate decarboxylase; 12, Alcohol dehydrogenase; 13, Glycerol phosphate dehydrogenase; 14, Glycerol phosphatase;15, Acetaldehyde dehydrogenase; 16, Galactokinase; 17, Galactose-1-phosphate uridylyltransferase; 18, Phosphoglucomutase; 19, UDP-galactose-4-epimerase; 20, Phosphomannose isomerase; 21, Phosphomannomutase; 22, Mannose-1-phosphate guanylyltransferase; Hxt, Hexose transporter; Gal2, Galactose permease (Madhavan et al., 2012).

To make the economics of any biomass conversion process cost-effective, the simultaneous and complete fermentation of all six- and five-carbon sugars present in the hydrolysates is essential (Madhavan et al., 2012). Although *S. cerevisiae*, is one of the most effective ethanol-producing microorganisms for hexose sugars including glucose, mannose, and galactose, the native strains are unable to utilize xylose for growth or fermentation.

Xylose-fermenting microorganisms are found among bacteria, yeast and fungi (Hahn-Hägerdal et al., 2006; Zaldivar et al., 2001). Thermophilic bacteria have also been considered for fermentation of pentose-containing lignocellulosic hydrolysates (Payton, 1984). Thermophilic and saccharolytic clostridia are an important group of ethanol-producing microorganisms and include species as *Clostridium thermohydrosulfuricum*, *Thermoanaerobacter* (formerly *Clostridium) thermosaccharolyticum* and *C. thermocellum* (Lovitt et al., 1988). The main drawback consists in their very low ethanol tolerance. In addition, the ethanolic fermentation occurs with considerable by-product formation, generally these bacteria produce mixed acids (acetate, lactate, propionate, succinate) and solvents (acetone, butanol, 2,3-butanediol), which reduce the ethanol yield (Saha, 2004). Yeasts as *Scheffersomyces stipitis, Scheffersomyces shehatae* and *Pachysolen tannophilus* can assimilate pentoses but their ethanol production rate from glucose is at least five times lower than that observed for *S. cerevisiae*. Moreover, their culture requires oxygen and ethanol tolerance is 2-4 times lower (Sanchez and Cardona, 2008). Moreover, these yeasts strains are inhibited by compounds generated during pretreatment and hydrolysis of the lignocellulose material (Hahn-Hägerdal et al., 2006). Filamentous fungi as *Orpinomyces* and *Piromyces* sp also can assimilate pentoses (Madhavan et al., 2009) but are too slow for a competitive industrial process (Hahn-Hägerdal et al., 2006; Matsushika et al., 2009a).

Natural xylose-fermenting yeasts, can metabolize xylose via the action of xylose reductase (XR) to convert xylose to xylitol, and of xylitol dehydrogenase (XDH) to convert xylitol to xylulose. Fig. 1.6 schematically illustrates the initial metabolic pathways for xylose utilization in fungi and bacteria. In most fungi and xylose-fermenting yeasts, D-xylose is converted to D-xylulose by two oxidoreductases involving cofactors NAD(P)H and NAD(P)$^+$. That is, D-xylose is initially reduced to xylitol by NAD(P)H-dependent xylose reductase (XR; EC 1.1.1.21), and then xylitol is oxidized to D-xylulose by NAD$^+$-dependent xylitol dehydrogenase (XDH; EC 1.1.1.9). Finally, xylulokinase (XK; EC 2.7.1.17) phosphorylates D-xylulose into D-xylulose 5-phosphate (X5P), which is further metabolized through the pentose phosphate pathway (PPP). As this reaction uses adenosine triphosphate (ATP), the reaction rate is dependent on the energy charge and the phosphorylation potential of the cell. On the other hand, in most bacteria and a few fungi D-xylose is directly isomerized to D-xylulose by xylose isomerase (XI; EC 5.3.1.5) also metabolized through the pentose phosphate pathway (PPP). D-xylulose is phosphorylated to D-xylulose 5-phosphate by XK (Matsushika et al., 2009a).

The genetic engineering and/or adaptation may be promising methods to develop sufficient xylose fermentation in *S. cerevisiae* (Chu and Lee et al., 2007; Matsushika et al., 2009a, 2009b) once that is xylose is by far the most abundant pentose sugar in lignocellulosic materials. Although *S. cerevisiae* cannot utilize xylose, the genes encoding XR (*YHR104w*, *GRE3*), XDH (*YLR070c*, *ScXYL2*), and XK (*XKS1*) are present in its genome; however, their expression levels are too low to allow for xylose utilization. In fact, only very slow growth on xylose has been observed even when the endogenous genes are over expressed in *S. cerevisiae*. Evolutionary engineering with *S. cerevisiae* conducted to improved ability of growing on xylose without heterologous genes (Çakar et al., 2005; Liu and Hu, 2010).

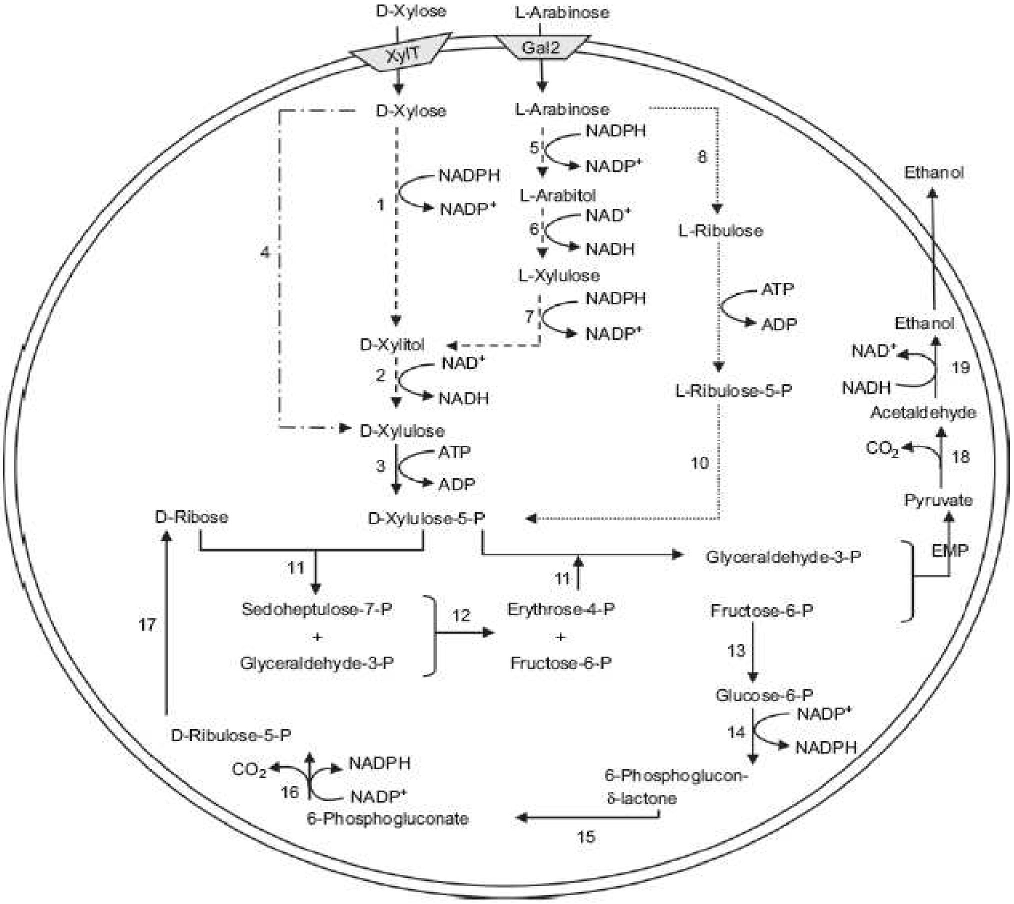

Figure 1.6 Metabolic routes for assimilation of the pentose sugars, xylose and arabinose by the oxidative Pentose Phosphate pathway. 1, Xylose reductase; 2, Xylitol dehydrogenase; 3, Xylulokinase; 4, Xylose isomerase; 5, Aldose reductase; 6, Arabitol dehydrogenase; 7, Xylulose reductase; 8, Arabinose isomerase; 9, Ribulokinase; 10, Ribulose-5-phosphate-4-epimerase; 11, Transketolase; 12, Transaldolase; 13, Glucose-6-phosphate isomerase; 14, Glucose-6-phosphate dehydrogenase; 15, 6-Phosphogluconolactonase; 16, 6-Phosphogluconate dehydrogenase; 17, Ribose-5-phosphate ketol-isomerase; 18, Pyruvate decarboxylase; 19, Alcohol dehydrogenase; XylT, Xylose transporter; Gal2, Galactose permease; EMP, Embden-Meyerhof-Parnas pathway comprising multiple steps. Dotted arrows represent bacterial pathways, dashed arrows represent fungal pathways and dash-dot arrows represent the XylA pathway existing in bacteria and some fungi (Madhavan et al., 2012).

A major focus in metabolic engineering for xylose fermentation has been in the area of establishing and improving an intracellular xylose-utilizing pathway in *S. cerevisiae* (Gírio et al., 2010; Hahn-Hägerdal et al., 2007). Two tasks are prerequisites for the synthesis of a *S. cerevisiae* strain that efficiently converts xylose to ethanol: (1) expression of heterologous genes should enable *S. cerevisiae* to convert xylose to xylulose; xylose isomerase or xylose reductase (XR) and xylitol dehydrogenase (XDH) and (2) consumption of xylulose should be improved since *S. cerevisiae* grows on xylulose with a maximum specific growth rate 10 times lower than that on glucose. Xylulose enters the pentose phosphate pathway (PPP) after phosphorylation by xylulokinase (XK), encoded by the XKS1 gene, which is naturally present in *S. cerevisiae*, but consequently a high capacity of the PPP is required (Ostergaard et al., 2000). Although the XR/XDH pathway generally presents unbalanced cofactor requirements and xylitol secretion, it is thermodynamically more advantageous than the xylose isomerase pathway, allowing faster xylose assimilation and ethanol production by engineered yeast strains (Karhumaa et al., 2007a, 2007b).

Several challenges faced by the yeast *S. cerevisiae* during the production of ethanol from lignocellulosic feedstocks have been object of study. Almost each process step for bioethanol production gives rise to environmental stress factors that may impact the ethanol yield or productivity in the fermentation such as the presence of organic acids (acetic, formic and levulinic), furaldehydes (furfural and HMF) and phenolic compounds (i.e. *p*-coumaric and ferulic acid) generated during the pretreatment (Almeida et al., 2007; Almeida et al., 2010). Moreover after pretreatment, the pH value must be adjusted, and this will lead to the formation of salt, which can cause osmotic stress for the yeast. The osmotic stress will trigger glycerol formation, affect membrane transport processes and slow down growth (Modig et al., 2007). In addition, the presence of highly contaminated feedstock and non-sterile conditions introduce an additional challenge of competition for the available sugars.

1.9.2 Industrial xylose-fermenting *Saccharomyces cerevisiae* strains

Industrial yeast strains tolerate many hydrolysates better than laboratory strains, which are why generating pentose-fermenting *S. cerevisiae* strains with an industrial background is necessary (Hahn-Hägerdal et al., 2007).

Whereas laboratory strains of *S. cerevisiae* are useful for evaluating metabolic engineering strategies, they do not possess the robustness required in the industrial context. Laboratory strains are usually haploid and have a defined genetic composition, and their genetic modification has been eased by the introduction of auxotrophic selection markers, which facilitates metabolic engineering strategies involving plasmids (Matsushika et al., 2009a). Furthermore, legal regulations and public perception will create obstacles to the acceptance of antibiotic selection markers on the industrial scale, even though these can be removed. Instead of plasmid usage, conserved and repeated genomic regions, such as ribosomal DNA (rDNA) sequences, can be useful targets for chromosomal gene integration in industrial yeast strains. In order to overexpress a given metabolic pathway, as the pentose assimilation pathway in *S. cerevisiae*, those regions are attractive to chromosomally integrate multiple gene copies. In contrast to the well-defined genetic make-up of laboratory strains, industrial strains are diploid, polyploid, or aneuploid, and often contain genetic segments from several yeast species (Matsushika et al., 2009b). The presence of multiple chromosome sets makes gene deletion a particular challenge because the target gene must be deleted from all chromosome alleles. However, genomic integration of genes encoding the pentose utilization pathway enzymes into industrial strains is relatively straightforward because positive selection for growth on xylose or arabinose can be used (Gírio et al., 2010). Only a limited number of industrial pentose-fermenting strains have been described in literature, and all genetic engineering has been limited to the introduction of the initial xylose and arabinose utilization pathways (Ostergaard et al., 2000). Further improvement of the recombinant strains has been achieved by adaptation strategies, including random mutagenesis, evolutionary engineering, and breeding (Çakar et al., 2005; Menon and Rao, 2012; Ostergaard et al., 2000).

Among the industrial *S. cerevisiae* the strain BH42 is one of the most attractive microorganisms for bioethanol production from lignocellulosic materials, due its high sugar consumption rate and ethanol yield in xylose medium when compared with other industrial *S. cerevisiae* xylose-fermenting (Sonderegger et al., 2004). This strain resulted from breeding of recombinant xylose-fermenting strains (Fonseca et al., 2004). Evolutionary engineering of *S. cerevisiae* TMB 3001, in continuous culture under microaerobic conditions, resulted in two mutant subpopulations with distinct and improved characteristics for xylose utilization. One of the resulting strains was able to grow in xylose under anaerobiosis. Breeding of those yeast strains (YIpXR/XDH/XK) in a mixture with other recombinant xylose-fermenting strains with industrial background resulted in strain *S. cerevisiae* BH42 (Fonseca et al., 2004; Gírio et al., 2010).

1.10 Hydrolysis and fermentation strategies for 2G bioethanol production

The classic configuration employed for fermenting biomass hydrolysates involves a sequential process where the hydrolysis of cellulose and the fermentation are carried out in different units. This configuration is known as separate hydrolysis and fermentation (SHF). In the alternative variant, the simultaneous saccharification and fermentation (SSF), the hydrolysis and fermentation are performed in a single unit (Sanchez and Cardona, 2008).

Other promising integration alternatives are simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP), known also as direct microbial conversion (DMC). The SSCF involves the fermentation of cellulose- and hemicelluloses-derived sugars in the SSF and the CBP combines cellulase production, cellulose hydrolysis and glucose fermentation into a single step (Cardona and Sanchez, 2007).

1.10.1 Separate hydrolysis and fermentation (SHF)

SHF is a conventional two steps process where the lignocellulose is hydrolysed using enzymes to form reducing sugars in the first step and the sugars, thus formed, are fermented to ethanol in the second step. The advantage of this process is that each step can be carried out at its optimum conditions, i.e. enzymatic hydrolysis at (45–50°C) and fermentation (28–35°C), furthermore, separate steps minimize undesired interactions (Lin and Tanaka, 2006; Menon and Rao, 2012; Sarkar et al., 2012). The disadvantages of this method are the product inhibition of cellulolytic enzymes, as the inhibition of β-glucosidase by glucose released during hydrolysis, which calls for lower solids loadings and higher enzyme loadings to achieve reasonable yields. Furthermore, the high sugar availability during the hydrolysis step increases the chances of contamination (Balat, 2011; Hahn-Hägerdal et al., 2006; Sarkar et al., 2012).

1.10.2 Simultaneous saccharification and fermentation (SSF)

In the SSF process the cellulolytic enzymes and microorganisms are added to the same process unit allowing that the glucose formed during the enzymatic hydrolysis of cellulose is immediately consumed by the microorganisms converting it into ethanol and relieving the inhibition of the enzymes by the end-product (Sanchez and Cardona, 2008). Furthermore, the fermentation seems to decrease the inhibition of the enzymes by converting some of the toxic compounds present in the hydrolysate (Hahn-Hägerdal et al., 2006; Lin and Tanaka, 2006). Moreover, the accumulation of ethanol in the reactor does not inhibit cellulolytic enzymes as much as the high concentration of glucose. Therefore, SSF is a good strategy for increasing the overall rate of cellulose to ethanol conversion. In comparison to the process where the two stages are sequential (SHF), SSF enables higher ethanol yields from cellulose, as well as eliminates the need for separate reactors for saccharification and fermentation. Other advantages of this approach are a shorter process time and a reduced risk of contamination with external microflora, due to the low sugar availability during the process and the presence of ethanol in the medium. In spite of the obvious advantages presented by the SSF, it has some drawbacks. These lie mainly in different optimal temperature for hydrolysis (45-50°C) and fermentation with *S. cerevisiae* (30-35°). The optimal temperature for SSF is around 35-38°C, which is a compromise between the optimal temperatures for hydrolysis and fermentation (Lin and Tanaka, 2006; Sun and Cheng, 2002) however microbial re-use is not possible since it is difficult to be separated from lignin. Summarizing, the SSF process shows more attractive indexes than the SHF as higher ethanol yields and less energetic consumption occur with improving of the bioethanol production economics.

1.10.3 Simultaneous saccharification and co-fermentation (SSCF)

SSCF has been recognized as a feasible option for ethanol production from xylose-rich lignocellulosic materials (Menon and Rao, 2012). The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the hemicellulose sugars to be converted to ethanol with SSF of cellulose. The SSCF process is considered to be an improvement to SSF. Therefore, only a single fermentation step is required to process both the liquid (hydrolysates) and solid fraction of the pretreated lignocellulose (Taherzadeh and Karimi, 2007). Co-fermentation of hydrolysis end-products glucose and xylose is reported to be carried out by genetically engineered microbes (Menon and Rao, 2012). For recombinant strain of *S. cerevisiae*, SSCF is of particular relevance for the co-fermentation of xylose, together with low glucose concentrations. Since *S. cerevisiae* does not harbour specific xylose transporters, xylose co-consumption in recombinant xylose-fermenting *S. cerevisiae* strains is only possible at low glucose concentrations, as the sugar transporters have higher affinity to glucose than to xylose (Olofsson et al., 2008). On the other hand, glucose has a positive effect on xylose fermentation, since xylose is not effectively recognized as a fermentable sugar, while glucose plays an important role in maintaining high glycolytic flux and repression of respiratory pathway.

1.10.4 Consolidated bioprocessing (CBP) or direct microbial conversion (DMC)

CBP combines the production of cellulolytic (and hemicellulytic) enzymes, cellulose (and hemicellulose) hydrolysis and glucose (and xylose/other sugars) fermentation into a single step. This process is attractive in that it reduces the number of reactors, simplifies operation, and reduces the cost of chemicals (e.g. commercial enzymes). CBP seems the logical endpoint in the evolution of bioethanol production from lignocellulosic materials. Application of CBP entails no capital investment for dedicated enzyme production, reduced diversion of substrate for enzyme production, and moves towards the compatibility of enzyme and fermentation systems (Balat, 2011). CBP requires a microbial culture that combines properties related to both substrate utilization and product formation. Desired substrate utilization properties include the production of a hydrolytic enzyme system allowing high rates of hydrolysis and utilization of resulting hydrolysis products under anaerobic conditions with a practical growth medium. Desired product formation properties include high product selectivity and concentrations. The disadvantages CBP systems are the low bioethanol yields, caused by by-product formation (acetate, lactate), low ethanol tolerance, and limited growth in hydrolysates (Balat, 2011).

To date, CBP for biofuel fermentation using genetically modified *S. cerevisiae* is an emerging technology that has been developed in several studies. These studies demonstrate that in addition to its co-fermentative genetic flexibility, *S. cerevisiae* can also be genetically engineered to express cellulolytic and hemicelluloytic heterologous enzymes. The successful functionality of cohesin and dockerin from *C. cellulolyticum* cellulosome in *S. cerevisiae* proved that this genetic modification based on a minicellulosome model may be an attractive option to the CBP process in hydrolyzing and fermenting substrates in a single step (Katahira et al., 2004).

1.11 Bioethanol recovery

As lignocellulose hydrolysis and fermentation technologies approach commercial viability, advancements in product recovery technologies are also required. For cases in which fermentation products are more volatile than water, recovery by distillation is often the technology of choice. Distillation technologies that will allow the economic recovery of dilute volatile products from streams containing a variety of impurities have been developed (Balat, 2011) and commercially demonstrated. A distillation system separates the bioethanol from water in the liquid mixture and is commonly used in commercial 1G ethanol plants and demonstration 2G ethanol plants.

The product stream from fermentation, so-called ‘‘beer’’, is a mixture of ethanol, cell mass, water, unfermented sugars and, in some cases of 2G technology, lignin. The first step is to recover the bioethanol in a distillation or beer column, where most of the water remains with the solid fraction. The product (approx. 37% bioethanol) is then concentrated in a rectifying column to a concentration just below the azeotrope (95%) (Balat, 2011). Hydrated ethanol (E95) can be employed in internal combustion engine vehicles (ICEVs), or in future fuel cell vehicles (FCVs) (requires onboard reforming), but for mixtures with gasoline water-free (anhydrous) ethanol is required. One can further distillate in the presence of an entrainer (e.g. benzene), dry by desiccants (e.g. corn grits), or use pervaporation or membranes. By recycling between distillation and dehydration, eventually 99.9% of the ethanol in the beer is retained in the dry product (Hamelinck et al., 2005).

To achieve an ethanol concentration of >4% (w/w), which is considered to be the benchmark for an efficient distillation, sugar levels of at least 8% (w/w) are needed, which implies an initial solids content of >15% (w/w) in the hydrolysis step, for most types of lignocellulosic biomass.

1.12 Current situation and perspectives for 2G bioethanol production in Europe

Several companies are now launching the first commercial lignocellulosic ethanol plants, although their viability is yet to be proven. Significant efforts in research, development and demonstration have been undertaken mostly in industrialized countries. In Europe, several research and development projects as well as pilot and demonstration plants on 2G bioethanol have been implemented and the first commercial plant was lauched in 2013, in Crescentino, Italy, by Biochemtex. Some of the major companies producing lignocellulosic ethanol in Europe are listed in Table 1.4.

The demonstration plant of Abengoa Bioenergy is located in Babilafuente in Salamanca (Spain). The plant had the capacity of processing 70 tons of agricultural residues per day. The pretreatment used is acid catalysed steam explosion, and the conversion technology is SHF (Abengoa Bioenergy, 2013).

Table 1.4 Pilot and demonstration plants of 2G bioethanol in Europe.

Operator	Location	Raw Material	Biomass processing capacity (t/yr)	Ethanol capacity (ML/yr)
Abengoa Bioenergy	Salamanca, Spain	Municipal solid wastes	25000	1.5
Biochemtex/Beta Renewables	Crescentino, Italy	Arundo donax and wheat straw	270000	50
Borregaard	Sarpsborg, Norway	Spruce wood	400	0.14
Clariant (Süd-Chemie)	Münich, Germany	Wheat straw or corn stover or sugarcane bagasse	4500	1.27
DTU, BioGasol	Copenhagen, Denmark	Wheat straw and bran, corn stover, energy crops and green wastes	20000	5
Inbicon, DONG Energy	Kalundborg, Denmark	Wheat straw	30000	5.4
Procethol 2G, Futurol	Pomacle, France	Wheat straw, switchgrass, green waste, Miscanthus	30000	3.5
SP (SEKAB)	Örnsköldsvik, Sweden	Spruce wood	700	0.2

Abengoa Bioenergy, 2013; Biochemtex, 2014; BioGasol, 2011; Borregard, 2014; Clariant, 2013; Futurol/Procethol 2G, 2014; Inbicon, 2014; SP, 2012.

The company BioGasol operates the Maxifuel pilot plant at the Technical University of Denmark. The company is developing one of the first Danish demonstration 2G ethanol plants. The process technology is a four-step SSF: (1) the pretreatment consists in a proprietary process based on a combination of steam-explosion and wet oxidation using both addition of oxygen and a pressure release at a temperature in the range of 179–200ºC; (2) a SSF is performed; (3) xylose is fermented using a proprietary thermophilic anaerobic bacterium; (4) the process water is converted in biogas and the water is reused. The Biorefinery Demonstration plant located in Örnsköldsvik, Sweden, has the capacity to process 2 tons of dry biomass per day. In addition of wood chips other lignocellulosic feedstocks such as sugarcane bagasse, wheat straw, corn stover and recycled paper has also been tested. The plant can operate with two options: (1) two stage dilute acid hydrolysis in either co-current or counter-current mode; (2) dilute acid pretreatment step followed by SSF (BioGasol, 2011).

In 2011 Süd-Chemie became part of Clariant. A demonstration plant of Clariant, located in Straubing-Münich (Germany) use the Sunliquid® technology for 2G ethanol production, which involves a hydrothermal pretreatment at mild process conditions, a process-integrated enzyme production, enzymatic hydrolysis, a specialized fermentation organism that simultaneously converts six and five-carbon sugars into ethanol (Clariant, 2013).

Biochemtex/Beta Renewables, located in Crescentino (Italy), uses the Proesa™ technology for 2G ethanol production. In this technology a two process pretreatment is performed followed by hydrolysis and fermentation accomplished in unique process (SSCF) yielding high ethanol concentrations (Biochemtex, 2014).

Inbicon, a subsidiary of the Danish DONG Energy, has the demonstration plant of 2G bioethanol located in Kalundborg (Denmark). The process technology adopted consists in a hydrothermal pretreatment, steam pretreatment containing acetic acid is used at 180-200ºC for 10-20 being able to operate under SHF or SSF modes. The heat is provided by a cogeneration plant that will use solid fuel based on the valorization of lignin. The surplus of electricity is sold to the electrical network (Inbicon, 2014).

1.12.1 Technological challenges of 2G bioethanol biorefineries

2G bioethanol biorefineries would simultaneously produce bioethanol, heat and power, as well as bio-based chemicals from biomass (Ghatak, 2011; Demirbas, 2009). The development of integrated biorefining technologies have been contributing to reduce operational cost of 2G bioethanol production and thus to its commercial deployment.

There are a lot of technical and non-technical barriers related to the implementation and commercialization of 2G bioethanol biorefineries. Although some significant progresses have been achieved, there are still some challenges to overcome.

The recalcitrance of lignocellulosic materials is due to the natural barrier of plant protection against external intrusion and degradation, which difficult the direct enzymatic hydrolysis of polysaccharides. Thus, the conversion of lignocellulosic materials generally requires pretreatment. The efficiency and the cost of pretreatment are key issues for the deployment of 2G bioethanol technology (Banerjee et al., 2010; Yang and Wyman, 2008) and this step has been the focus of research and the core business of several industrial players. The goal is to significantly improve the performance of the enzymatic hydrolysis of cellulose and, at the same time, do not generate significant amount of inhibitors for the bioconversion steps (Alvira et al., 2010).

Also, the efficiency and the cost of the enzymatic hydrolysis step still require improvements. The hydrolysis of cellulose is generally very efficient, even at high solid loading, as the most advanced cocktails have hydrolytic enzymes subject to low inhibition by the product. The enzyme manufacturers have significantly reduced the cost of enzyme production, but it still contributes significantly to the operational costs of bioethanol production. Therefore, it is important to reduce enzyme loading without loss of performance. Additionally, the performance of commercial cellulolytic cocktails still depends of the feedstock and pretreatment used and the research trends are now on the development of tailored cocktails for specific combination of feedstock/pretreatment. Other factors affecting enzyme performance which requires attention are enzyme stability (e.g. to temperature) and to the present of putative inhibitors, both feedstock-specific and/or generated during pretreatment (Koppram et al., 2013; Kumar et al., 2009; Viikari et al., 2012).

The biochemical conversion of lignocellulose into bioethanol also requires robust fermenting organisms, able to ferment the main constituent monosaccharides of lignocellulose (usually hexoses and pentoses), in hydrolysates containing inhibitors, at high ethanol concentration, yield and productivity (Banerjee et al., 2010; Hahn-Hägerdal et al., 2006; Viikari et al., 2012).

Moreover, the success of the 2G bioethanol technology at industrial level requires optimal process integration in order to reduce the number of process steps and the related equipment and energy demand, and to re-use process streams, ultimately achieving zero-waste and significantly reducing water consumption.

Concerning the non-technical barriers, these involve aspects related mainly to the feedstock availability and supply. One of the critical challenges in the biorefineries supply chains is the seasonal nature and annual variability of biomass supply. Moreover, the harvesting timing and frequency may affect the composition of the biomass. The continuous and steady demand for transportation fuels throughout the year requires a continuous and steady biomass supply in 2G bioethanol biorefineries. The operational challenge is to manage the biomass storage in order to maintain a continuous supply. However, the cost associated to storage promotes an increase of the product price, which is magnified by biomass degradation due to long storage periods. The feedstock price, including logistics is represents a significant cost associated to 2G bioethanol biorefineries. Therefore, a suitable design of the logistics is one of the key factors for keep the cost of feedstock low (EERA, 2013; Yue et al., 2014).

1.13 Innovation and Objectives

Constrains related to biomass supply in biorefineries, including seasonality, availability, and storage, can be overcome by the use of feedstock mixtures rather than individual feedstocks. A few works have been conducted processing mixtures of lignocellulosic materials for ethanol production but, in general the same type of feedstocks are used (e.g. agricultural residues).

The relatively low biomass availability and the seasonality of several feedstocks is a drawback for the continuous operation of bioethanol biorefinery in Southern Europe. Therefore, the use of mixtures of lignocellulosic materials, like agricultural residues (e.g. wheat straw) and hardwoods residues (e.g. eucalyptus residues and olive tree pruning) can contribute to overcome biomass supply limitations for bioethanol biorefineries in this region. Moreover, the processing of mixtures containing different proportions of each feedstock can overcome seasonality of some feedstocks and contribute to the development of an efficient system of biomass supply for larger scale biorefineries.

Thus, this work pursued the following objectives:

- The study of hydrothermal pretreatment (autohydrolysis) to increase cellulose digestibility and to selective fractionate hemicelluloses in mixtures of lignocellulosic materials, particularly eucalyptus residues (ER), composed by bark, branches and leaves, wheat straw (WS) and olive tree pruning (OP).
- The optimization of enzymatic hydrolysis conditions of pretreated solids of ER, WS and OP and their mixtures, studying the effect of pretreatment temperature, enzyme dosage and solid loading.

- The co-fermentation of glucose and xylose, obtained from processing the different mixtures of ER, WS and OP through pretreatment and enzymatic hydrolysis, using a recombinant xylose-fermenting *Saccharomyces cerevisiae* strain under different process configurations for 2G bioethanol production.

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CHAPTER

2

HYDROTHERMAL PRETREATMENT

PART A - Biorefining strategy for maximal monosaccharide recovery from three different feedstocks: eucalyptus residues, wheat straw and olive tree pruning

Abstract

This work proposes the biorefining of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) combining hydrothermal pretreatment (autohydrolysis) with acid post-hydrolysis of the liquid fraction and enzymatic hydrolysis of the solid fraction towards maximal fractionation of lignocellulose components and recovery of monosaccharides. Autohydrolysis of ER, WS and OP was performed under non-isothermal conditions (195-230ºC) and the non-cellulosic saccharides were recovered in the liquid fraction while the cellulose and the lignin virtually remained in the solid fraction. The acid post-hydrolysis of the soluble oligosaccharides was studied by optimizing sulfuric acid concentration (1-4% w/w) and reaction time (10-60 min), employing a factorial (2^2) experimental design. The solids resulting from pretreatment were submitted to enzymatic hydrolysis by applying commercial cellulolytic enzymes Celluclast 1.5L and Novozyme 188 (0.225 and 0.025 g/g_{solid}, respectively). The strategy proposed can be effectively applied to the fractionation and maximal monosaccharide recovery from the three selected materials.

Keywords

Lignocellulosic materials, sugar-based biorefinery, autohydrolysis, enzymatic hydrolysis, chemical post-hydrolysis

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Highlights

- Eucalyptus residues, wheat straw and olive tree pruning are suitable for Southern European biorefineries.
- The proposed biorefining strategy is efficient for monosaccharide recovery.
- Maximal monosaccharide recovery was obtained after autohydrolysis at 210ºC.
- Maximal cellulose enzymatic digestibility was obtained after autohydrolysis at 230ºC.
- The optimal biorefining conditions for maximal monosaccharide recovery were similar for all feedstocks.

Abbreviations

OS, oligosaccharides, GlcOS, glucooligosaccharides; XOS, xylooligosaccharides; AOS, arabinooligosaccharides; GalOS, galacto-oligosaccharides; MOS, manno-oligosaccharides; HMF, 5-(hydroxymethyl)furfural; X_1, sulfuric acid concentration; X_2, isothermal reaction time.

2.1 Introduction

Lignocellulosic materials are the largest renewable and potentially sustainable source of biomass that can be used in a biorefinery framework for the production of fuels, chemicals and materials (FitzPatrick et al., 2010; Ghatak, 2011). ER, WS and OP are lignocellulosic materials largely available in Southern Europe (Faraco and Hadar, 2011) and should thus be considered in the biomass supply chain for biorefineries within this region. Moreover, the availability of these materials varies throughout the year, which can contribute to a steady biomass supply for continuous biorefining in this region.

Eucalyptus is distributed worldwide, in countries like Brazil, China and India, but the species *Eucalyptus globulus*, which generates high-quality pulp and paper, is found mainly in Australia, Portugal and Spain. *E. globulus* is usually managed in short rotations (10-12-year long) and the biomass collected after thinning is considered a non-seasonable renewable raw material as it is produced throughout the year. Wheat is also widely distributed and the main wheat producers are China, India, US, Russia and Europe in general. Wheat is an annual, rain-fed crop predominantly cultivated in autumn and harvested in late spring/early summer, being the straw available at this time. In turn, olive tree is concentrated in the Mediterranean region and is one of the most important agricultural crops in Southern Europe. It requires pruning in intervals of 1 or 2 years, being performed when the vegetative activity of the olive is minimum, i.e., winter and early spring. Thus, the relative complementary seasonality of these three lignocellulosic materials in Southern Europe can allow the predominant use of a given raw material regarding the other two, in the period in which it is available in more abundance.

Due to their high saccharide content the upgrading of ER (Canettieri et al., 2007), WS (Talebnia et al., 2010) and OP (Romero-García et al., 2014) is thought to be more effective if carried out through a sugar platform for the (bio)chemical production of biofuels or other bio-based products (Duarte et al., 2007; FitzPatrick et al., 2010). Biochemical conversion processes of lignocellulosic biomass usually require prior fractionation processes to obtain the individual components (cellulose, hemicellulose and lignin) and thus increase their value (Demirbas, 2009). Hydrothermal treatments, namely autohydrolysis, have been described as effective pretreatment methods, with the advantages of enabling the selective removal of hemicelluloses, together with a virtual complete recovery of cellulose and lignin in the solid phase (Carvalheiro et al., 2009; Gírio et al., 2010; Ruiz et al., 2013). This feature turns autohydrolysis an adequate pretreatment for the development of integrated biorefining strategies aiming at the fractionation and hydrolysis of cellulose and hemicellulose into monomeric sugars. Furthermore, autohydrolysis mainly yields soluble OS in the liquid phase, which are added-value compounds as they present many relevant traits for the feed, food, and pharmaceutical industries (Carvalho et al., 2013). Nevertheless, their market volume is still low and cost-effective biorefining process may require the utilization of all sugars available from cellulose and hemicellulose, as in the case of lignocellulosic ethanol (Gírio et al., 2010). Oligosaccharides can thus integrate the biorefinery sugar-stream by a post-hydrolysis step, using acids or enzymes to obtain monosaccharides. Previous results have demonstrated that, for this purpose, dilute-acid post-hydrolysis is an efficient approach (Duarte et al., 2009; Garrote et al., 2001; Gírio et al., 2010), while hemicellulose enzymatic hydrolysis requires a synergy between several enzymes for complete conversion into monosaccharides (Wyman et al., 2004). Moreover, since hemicelluloses may act as a physical barrier to cellulose breakdown by enzymes (Zhang et al., 2012) and XOS are strong inhibitors of cellulase (Yang et al., 2011), the enzymatic hydrolysis of cellulose is substantially improved by the extensive removal of hemicellulose promoted by autohydrolysis pretreatment.

For the complete recovery of monosaccharides from biomass, the cellulose fraction obtained in the pretreated solid requires hydrolysis into glucose (Ballesteros, 2010; Sun and Cheng, 2002). This hydrolysis may be accomplished by physical, chemical and/or biochemical processes. Here, enzymatic hydrolysis is advantageous in relation to acid hydrolysis due to: high specificity, minimal sugar degradation and by-products formation; lower energy consumption, as a result of milder reaction conditions; lower investment costs and maintenance of equipment (Ballesteros, 2010; Yang et al., 2011).

Accordingly, in this work, ER, WS and OP were subjected to an integrated biorefining strategy (Fig. 2.1) for fractionation and maximal sugar recovery involving: 1) an autohydrolysis step in order to selectively solubilize the hemicellulose fraction; 2a) a chemical post-hydrolysis step of oligosaccharide-rich hydrolysates (liquid fraction from autohydrolysis) towards maximal recovery of hemicellulosic monosaccharides; 2b) an enzymatic hydrolysis of the cellulose (solid fraction from autohydrolysis) towards maximal glucose recovery. The conditions of each step and their integration for fractionation of lignocellulose and maximal sugar recovery are discussed towards the deployment of flexible sugar-based biorefineries.

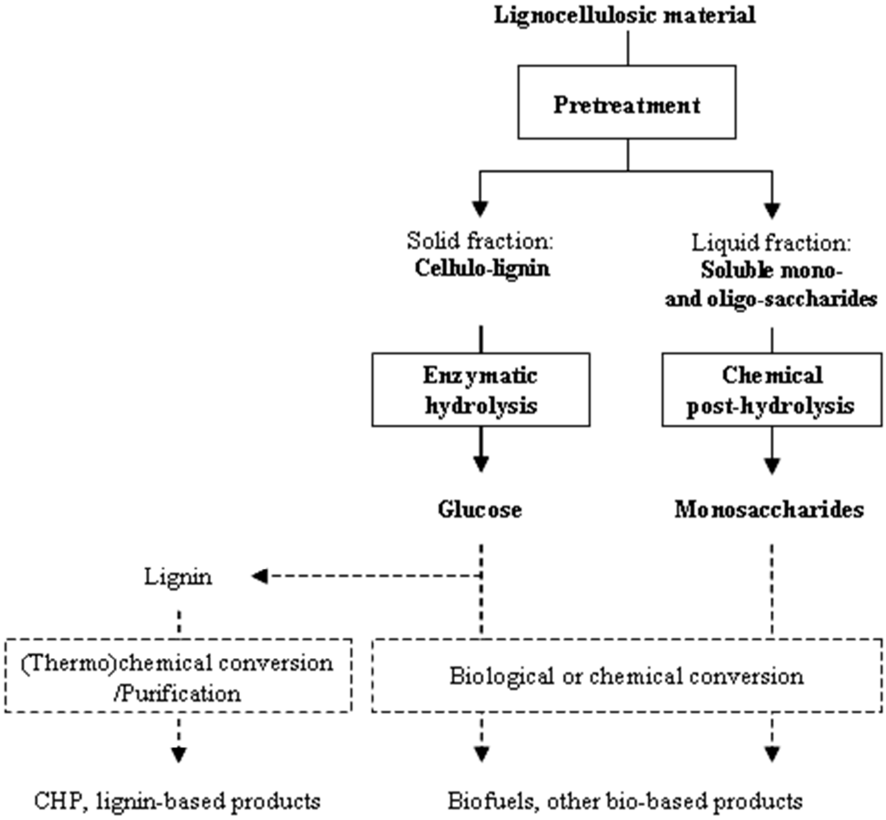


Figure 2.1 Proposed strategy for the fractionation of lignocellulosic biomass into monomeric sugars (pentoses and hexoses) and lignin.

2.2 Materials and Methods

2.2.1 Raw materials

Eucalyptus residues (ER), consisting of bark, branches and leaves, wheat straw (WS) and olive tree pruning (OP), consisting of leaves and thin branches were collected from Mortágua, Elvas, and Ferreira do Alentejo, (Portugal), respectively. ER and OP were dried at 40ºC (to reach moisture content under 10% w/w) prior to usage. All the materials were individually ground with a knife mill (to particles smaller than 6 mm), homogenized, chemically characterized (as described below) and stored at room temperature, until usage.

2.2.2 Autohydrolysis

The autohydrolysis assays were performed in a stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) with an internal volume of 600 mL. The reactor was fitted with two four-blade turbine impellers, heated by an external fabric mantle, and cooled by water circulating through an internal stainless steel loop. Temperature was controlled through a Parr PID controller, model 4842. The different materials were mixed with water in a liquid-to-solid ratio of 7:1 (g water:g dry biomass). The agitation speed was set at 150 rpm and the reactor was heated to reach the final defined temperatures in the range of 195 to 230°C. After reaching the desired temperature, the reactor was rapidly cooled, by water circulation through a serpentine coil and its subsequent immersion in an ice bath. The liquid and solid fractions were separated by pressing (up to 200 bar) using a hydraulic press (Sotel, Portugal). The liquid fraction was further filtered through Whatman no. 41 filter paper and the solid fraction was washed with water (with a volume equivalent to twice the volume used in autohydrolysis) at room temperature, filtered and dried at 40°C, and its composition determined by quantitative acid hydrolysis as described below.

2.2.3 Optimization of the chemical post-hydrolysis of the liquid fraction from autohydrolysis

Chemical post-hydrolysis of the liquid fraction from autohydrolysis of ER, WS or OP (autohydrolysis temperature of 210ºC) was optimized by studying the effects of sulfuric acid concentration (X_1) between 1.0 and 4.0% (w/w), coded as (-1) and (+1) respectively, and isothermal reaction time (X_2) between 10 and 60 min, coded as (-1) and (+1) respectively, using a 2^2 factorial experimental design (Rodrigues and Iemma, 2009). In the central point, coded as (0), X_1 and X_2 is 2.5% sulfuric acid and 30 min, respectively. Each condition was assayed in duplicate. Sulfuric acid (72% w/w) was added at different ratios to obtain the prescribed acid concentration taking a constant total initial mass of 15 g.

The chemical post-hydrolysis reactions were carried out in an autoclave (A. J. Costa, Portugal), at 121°C, in universal 50-mL Schott flasks capped with stoppers. When the reaction time was attained, the autoclave was rapidly cooled down to 100°C.

The model used to express the responses is described in Equation 1, where: Y is the response, X_i the independent variables, and the subscripts 1 and 2 are referred to sulfuric acid concentration and time, respectively.

$$Y=\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \varepsilon \qquad \text{(Eq. 1)}$$

β_0 is the regression coefficient at center point; β_1 and β_2 are the linear coefficients of the variables 1 and 2, respectively (main effects); β_{12} is the coefficient of interaction between variables 1 and 2 (interaction effect), and ε are independent random errors, assumed to be normally and independently distributed. The linear regression to Eq. 1 and its analysis of variance (ANOVA) were carried out using Microsoft® Excel 2010 regression tool pack. The best hydrolysis conditions were determined by using the Microsoft Excel® 2010 Solver tool based on the best-fit equations using a constrained model. Coded representation of the variables was used for all calculation purposes.

2.2.4 Enzymatic hydrolysis of the solid fraction from autohydrolysis

The solid fraction resulting from autohydrolysis (at 210 and 230ºC) was suspended in 0.05 M sodium phthalate buffer (pH 5.5), at an initial concentration of 5.0% (w/v). The solids were incubated with a formulation of two commercial cellulolytic enzyme preparations, Celluclast® 1.5L

(0.225 g/g_{solid}) supplemented with Novozyme® 188 (0.025 g/g_{solid}) (corresponding to approx. 8 FPU/g_{solid}, and 24 U β-glucosidase/g solid), in an orbital shaker (ERTAL HT), at 140 rpm and 50ºC, for 48 h, in presence of 0.08% sodium azide to avoid biological contamination. The hydrolysates obtained after residual solid removal by centrifugation (13,000 rpm for 10 min) (Beckman Coulter™ Microfuge® 18) were incubated at 100ºC for 10 min to inactivate the enzymes and their monosaccharide composition analyzed by HPLC as described below.

2.2.5 Analytical methods

2.2.5.1 Chemical characterization of lignocellulosic materials and processed solids

The materials were ground in a knife mill (to a particle size <0.5 mm) and their moisture content was determined by oven-drying at 105°C to constant weight. The different materials, as well as the processed solids, were characterized for glucan, xylan, galactan, arabinan, mannan, acetyl groups and Klason lignin by quantitative acid hydrolysis, with 72% (w/w) sulfuric acid for 60 min at 30ºC, followed by dilution to 4% (w/w) sulfuric acid and hydrolysis for 60 min at 121ºC. The assays were performed in triplicate. Monosaccharides and acetic acid present in the hydrolysates were analyzed by HPLC as described below. Lignin content was determined from the remaining dried solids correcting for ash content, which was determined by igniting the solids at 550ºC for 5 h. Protein quantification was performed by the Kjeldahl method using the N x 6.25 conversion factor. Fat quantification was performed according to the Portuguese standard NP-876. Soluble monosaccharides were extracted according to the National Renewable Energy Laboratory (TP-510-42619) and analyzed and quantified by HPLC as described below. Starch was assayed by an enzymatic method with the Total Starch kit (K-TST 07/11, Megazyme). Total phenolic compounds content was assayed spectrophotometrically at 765 nm by Folin–Ciocalteu method (Singleton et al., 1999) using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific, MA, USA). Gallic acid was used as calibration standard.

2.2.5.2 HPLC characterization of liquid fraction from quantitative acid hydrolysis, autohydrolysis, chemical post-hydrolysis and enzymatic hydrolysis

The monosaccharides (glucose, mannose, galactose, xylose and arabinose), organic acids (formic, acetic, and levulinic acids) and furans (HMF, and furfural) were quantified by high-performance liquid chromatography (HPLC), using either an Aminex HPX-87H column from Bio-Rad (Hercules, CA) for quantifying monosaccharides, organic acids and furans, and a HPX-87P for further refine monosaccharides characterization and quantification. It was used an Agilent 1100 Series HPLC system (Germany) equipped with a refractive index (IR), controlled at 45ºC, to detect monosaccharides and organic acids, and a diode array detector (DAD) to detect furans. With the HPX-

87P column, the mobile phase was ultrapure water, the column temperature was set at 80°C and the flow rate and injection volume were set at 0.6 mL/min and 20 μL, respectively. With the HPX-87H, the mobile phase was 5 mM H_2SO_4 and the column temperature was set at 50°C. To analyze the samples from quantitative acid hydrolysis, the flow rate and injection volume was set at 0.4 mL/min and 20 μL, respectively. To analyze the liquid fraction from autohydrolysis, chemical post-hydrolysis and enzymatic hydrolysis, the flow rate and injection volume was set at 0.6 mL/min and 5 μL, respectively. All samples were filtered through 0.22 μm membranes before analysis. The concentration of OS in the liquid fraction from autohydrolysis was calculated from the increase in the concentration of monosaccharides as analyzed by HPLC after post-hydrolysis at standard conditions (corresponding to addition of sulfuric acid at an overall concentration of 4% (w/w) during 60 min at 121ºC) (Carvalheiro et al., 2004).

2.3 Results and Discussion

2.3.1 Composition of lignocellulosic materials

The lignocellulosic materials selected for this study, ER, WS and OP, presented high saccharide content (57-68%), accounting glucan (cellulose and starch), xylan, galactan, arabinan, mannan and extractable sugars (Table 2.1). The cellulose content was estimated based on glucan assessed by quantitative acid hydrolysis (as glucose) and subtracting starch and glucose in extractives. Taking into account this assumption, ER presented the higher cellulose content (36.1%), followed by WS (30.6%) and OP (24.5%).

However, the glucan content (expressed as glucose) corresponded to 40.7, 38.6 and 33.6% in ER, WS and OP, respectively. The differences observed have resulted from the high amount of soluble glucose and/or starch content in the WS and OP. In fact, the results obtained for total glucan (as glucose) and cellulose of OP were very similar to those previously reported in non-extracted raw material (32.7/32.9% and 22.7/25.0 respectively) (Ballesteros et al., 2011; Cara et al., 2008a). WS, which presented the highest starch content among the lignocellulosic materials tested (3.1%) but lower soluble glucose content (1.2%) than OP, presented a total glucan content similar to previously reported values (37.8-38.9%) in non-extracted raw material (Gírio et al., 2010; Pérez et al., 2008). The ER characterization previously reported pointed for approx. 40% of cellulose in extracted solids (Canettieri et al., 2007). In this study, the cellulose or total glucan content estimated for ER extracted solids accounted for approx. 41%. The cellulose content of ER was lower than previously reported for *Eucalyptus globulus* wood chips (around 46%) (Martín-Sampedro et al., 2012).

Table 2.1 Chemical composition of the eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) (% of dry weight).

Component	ER	WS	OP
Cellulose[a]	36.1 ± 0.6	30.6 ± 0.1	24.5 ± 0.2
Hemicellulose	23.8	32.3	18.8
Xylan	13.9 ± 0.8	23.5 ± 0.2	9.9 ± 0.1
Galactan	1.3 ± 0.1	0.7 ± 0.0	1.8 ± 0.5
Arabinan	2.0 ± 0.6	4.6 ± 0.3	3.2 ± 0.2
Mannan	1.7 ± 0.0	0.0	1.0 ± 0.1
Acetyl groups	4.9 ± 1.4	3.5 ± 0.2	2.9 ± 0.3
Klason lignin	26.7 ± 0.3	16.8 ± 0.2	22.2 ± 0.2
Ash	2.4 ± 0.3	5.0 ± 0.0	3.8 ± 0.1
Protein	2.8 ± 0.5	5.4 ± 0.0	6.2 ± 0.1
Extractives	3.6	9.8	18.6
Fat	1.9 ± 0.3	1.4 ± 0.0	4.3 ± 0.1
Starch	0.0	3.1 ± 0.5	2.3 ± 0.1
Soluble saccharides	1.7 ± 0.1	5.3 ± 0.7	12.0 ± 0.2
Glucose	0.6 ± 0.1	1.2 ± 0.0	3.8 ± 0.0
Others	4.6	0.1	5.9

[a] Calculated subtracting starch and free glucose from total glucan.

WS, the agricultural residue used in this study, presented the highest hemicellulose content (32.3%), which was higher than determined in other studies (25.4 and 23.5%) (Gírio et al., 2010; Pérez et al., 2008). Also, the hemicellulose content in ER (23.8%) was higher than reported for other eucalyptus residues and wood, 18 and 19.9%, respectively (Canettieri et al., 2007; Martín-Sampedro et al., 2012). The hemicellulose content of OP was very similar to that observed in non-extracted raw material by other authors (18.2%) (Ballesteros et al., 2011; Cara et al., 2008a). In WS, xylose and arabinose were the major hemicellulosic units, which is in agreement with the arabinoxylan nature of hemicellulose found in WS and other agricultural residues (Gírio et al., 2010). The hemicellulose of ER and OP was also mainly composed of xylan. Other hemicellulosic units, such as arabinose, galactose and mannose, were identified in lower amounts. Acetyl groups represented about 4.9, 3.5 and 2.9% of the ER, WS and OP, respectively.

Klason lignin also constituted a significant fraction of the raw materials studied, being over 20% in ER and OP, hardwood materials, and about 15% content in WS. Insoluble lignin content in WS (16.8%) was similar to that found by (Pérez et al., 2008), 16.3%, and slightly lower that reported by (Carvalheiro et al., 2009), 18.0%. The lignin content found in ER was similar to the previously reported (26%) (Canettieri et al., 2007), while the OP studied presented a higher value than the reported by other authors (16.6%) (Ballesteros et al., 2011; Cara et al., 2008a).

In the present work, the extractives were characterized as extractable monosaccharides, fat and starch. OP was the feedstock that presented the higher amount of extractives reaching a total of 18.6%, from which approximately 2/3 are saccharides. Other authors found higher extractives content in OP (23-31%) containing around 8% glucose (Cara et al., 2008a; Ballesteros et al., 2011). For the extractives of the other materials, a similar value was found by (Pérez et al., 2008) for WS (15.3%), and a lower value was obtained by (Romaní et al., 2011) for eucalyptus wood (2.4%). The ash content was low in the three lignocellulosic materials used in this work and the same was observed by other authors (Ballesteros et al., 2011; Canettieri et al., 2007). The exception was observed for WS, where (Carvalheiro et al., 2009) detected almost 10% of ash. This variability of the results could be attributed to the variety, the harvesting period and the heterogeneity of the feedstock.

2.3.2 Autohydrolysis as the fractionation process

Autohydrolysis was used as the pretreatment process for the selective fractionation of non-cellulosic from cellulosic sugars, with hemicellulose mainly recovered in the liquid fraction and the cellulose retained, as cellulo-lignin, in the solid fraction.

2.3.2.1 Liquid fraction

The composition of the liquid fraction obtained after autohydrolysis of ER, WS and OP in terms of OS, monosaccharides and degradation products was characterized as a function of pretreatment temperature (195-230ºC) (Fig. 2.2). The saccharides present in higher concentration were found to be the XOS, which resulted from the solubilisation of xylan, the major component of the hemicelluloses present in the three lignocellulosic materials tested. The autohydrolysis of ER, WS and OP generated a similar profile of non-cellulosic saccharides, being 210-215ºC the optimal temperature range for maximal sugar recovery in the liquid fraction in all the three materials tested. The composition of the liquid fraction of ER, WS and OP subject to an autohydrolysis process seems to be strictly related to the different composition of materials *in natura*. The liquid fraction obtained from WS autohydrolysis presented the highest concentration of XOS, reaching 20.5 g/L (15.2 g/100 g feedstock), while the pretreatment of ER and OP generated 12.0 g/L (9.3 g/100 g feedstock) and 10.3 g/L (7.6 g/100 g feedstock) of XOS, respectively. As such, more than 65% of the initial xylan was recovered as soluble XOS, after autohydrolysis at 210ºC of each raw material.

GlcOS were also obtained in relatively high concentrations in the liquid fraction of pretreated WS and OP, but not when ER was used. This is mainly correlated with the presence of starch in WS and OP (see Table 2.1). In both cases, the GlcOS recovery was high at autohydrolysis temperatures below 220ºC. The pretreatment of WS generated more than 9 g/L of GlcOS, which corresponded to almost half of the highest concentration of XOS obtained with this feedstock. This high GlcOS obtained from WS pretreatment contrasts with previously reported data (Carvalheiro et al., 2009), which can be partially justified by the different harvesting season and by the presence of residual amounts of starch grain in WS, which was now identified as 3.1% of total dry matter. In the liquid fraction of pretreated OP, a similar concentration of GlcOS and XOS (9.8 and 10.3 g/L, respectively) was observed at 210ºC. A similar characterization of the liquid fraction from OP autohydrolysis was previously described (Cara et al., 2012) and the GlcOS content was attributed to non-structural glucose issued from the extractive fraction of the raw material, rather than from solubilisation of cellulose. A different profile was observed in the liquid fraction of pretreated ER, where GlcOS were found in minor concentrations (maximum of 2.1 g/L, at 195ºC). This low GlcOS content confirmed the limited removal of structural glucan by autohydrolysis, which in the case of ER could represent partial or total solubilisation of the low amount of glucomannans present in the ER hemicellulose. Low concentrations of GlcOS (0.4-1.0 g/L) were also detected after autohydrolysis of *E. globulus* wood (Romaní et al., 2011).

Other OS obtained from hemicellulose hydrolysis such as AOS, GalOS and MOS were also present in the hydrolysates but, in general, at minor concentrations, with maximum AOS found in WS and OP (3.5-3.7 g/L) and GalOS in ER (3.4 g/L), at 195ºC.

Regardless the lignocellulosic material studied, the liquid fraction of autohydrolysis displayed a low concentration of monosaccharides under the tested conditions. Pentoses were the predominant monosaccharides obtained from pretreated ER and WS, whereas glucose was the most abundant monomer obtained from OP, in agreement with the high non-structural glucose content of this material. Arabinosyl units were significantly hydrolyzed at the lower temperatures tested (195-210ºC), while xylose was generated at maximal concentrations with the autohydrolysis performed at 215-220ºC. A similar profile was previously observed for WS (Carvalheiro et al., 2009), which is in agreement with a hemicellulose composed of a xylan backbone, more recalcitrant, and side chains containing arabinosyl, i.e. arabinoxylan.

The organic acids concentration in the liquid fraction increased steadily with the temperature of autohydrolysis (Fig. 2.2). Acetic acid was the main organic acid found in hydrolysates reaching the highest concentration (5 g/L) at 230ºC. Formic acid, which is formed when furfural and HMF are broken down (Palmqvist and Hahn-Hägerdal, 2000) was obtained at slightly lower concentrations (3.4 g/L, 4.5 g/L and 4.2 g/L at 230ºC, for ER, WS and OP, respectively). Similarly, phenolic compounds obtained from ER and WS also increased with autohydrolysis temperature. Conversely, the temperature appears to have little influence on the phenolic compounds concentration in the liquid fraction of pretreated OP at 215-230ºC.

Sugar degradation products (furfural and HMF) also increased with pretreatment temperatures, as generally observed in the pretreatment of lignocellulosic materials (Garrote et al., 1999). However, high furan concentrations (>4 g/L) were only obtained with autohydrolysis at 230°C. Furfural was obtained in higher concentrations than HMF, which is related to the more labile nature of pentoses and their higher concentrations. The highest concentration of furfural and HMF were found in WS (5.11 g/L and 0.77 g/L, respectively, at 230ºC).

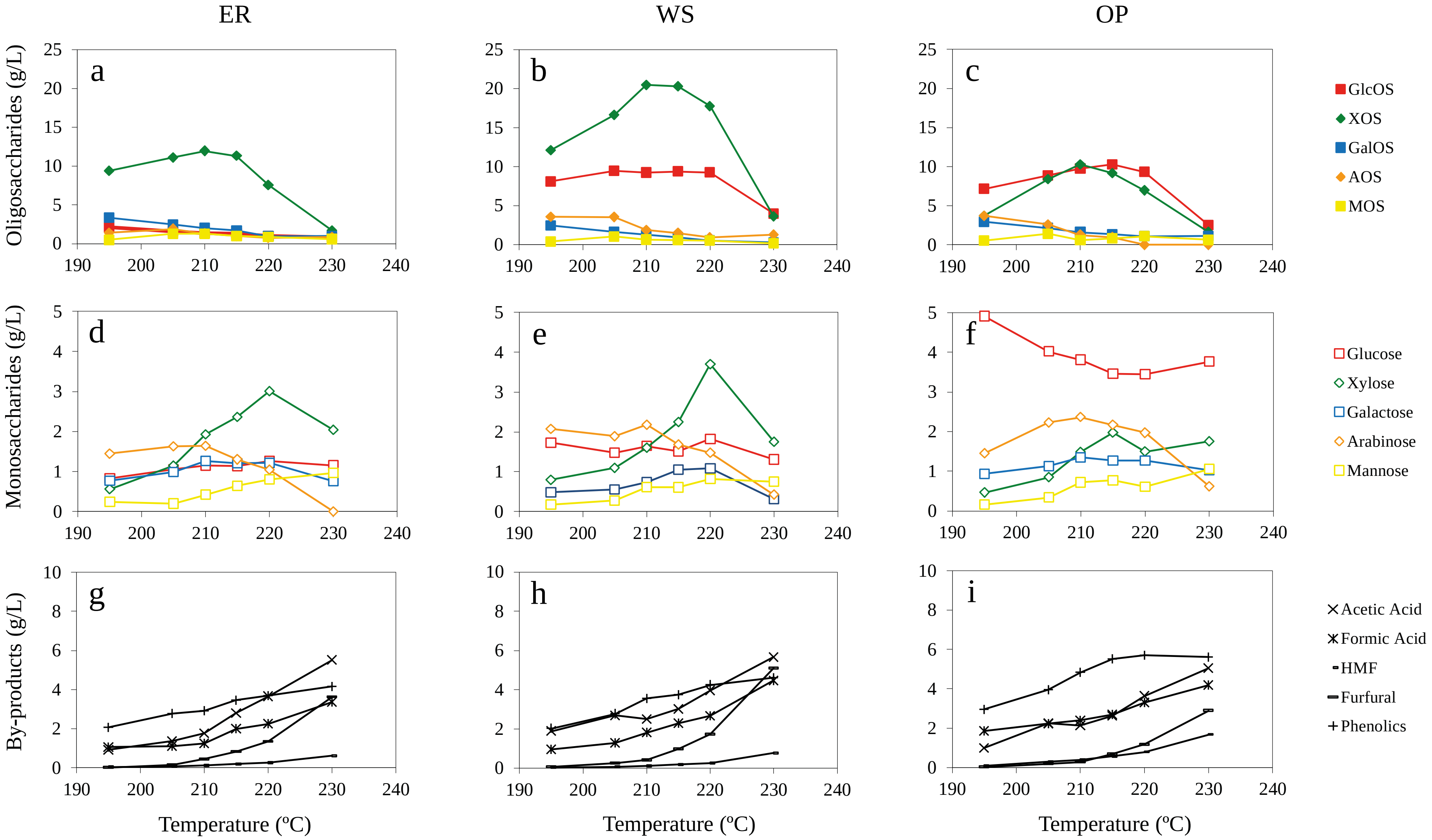


Figure 2.2 Composition of the liquid fraction obtained from autohydrolysis of eucalyptus residues (a, d, g), wheat straw (b, e, h) and olive tree pruning (c, f, i).

2.3.2.2 Solid fraction

The composition of the solid fraction of ER, WS and OP recovered after autohydrolysis at different temperatures (195-230ºC) is represented in Fig. 2.3. Hemicellulose content decreased with the increase of pretreatment temperature, what can be explained by the higher solubilization of its constituents. This is in agreement with the increasing sugar content of the liquid fraction with temperature (up to a point where significant degradation products were generated), and also with previous observations (Cara et al., 2008a; Carvalheiro et al., 2009).

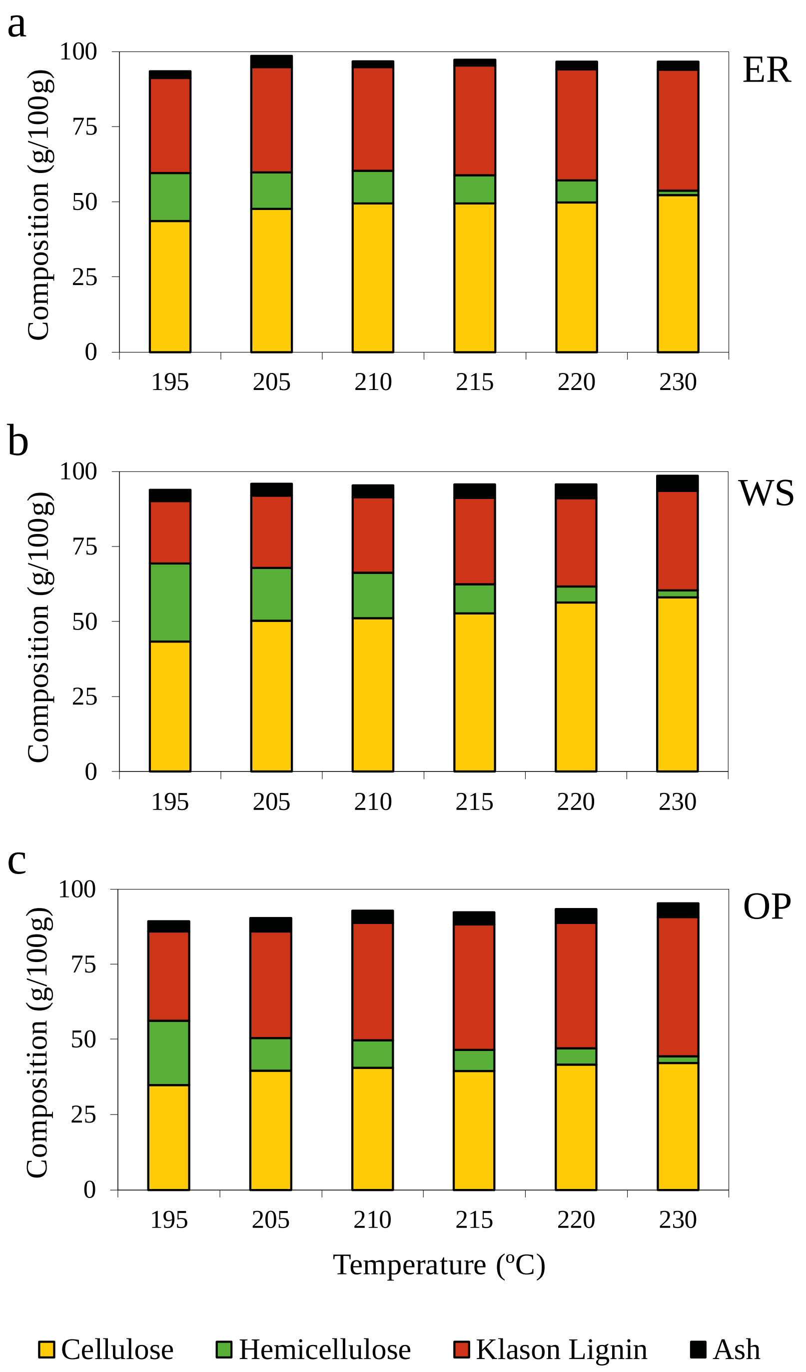


Figure 2.3 Composition of the solid fraction recovered after autohydrolysis of eucalyptus residues (a), wheat straw (b) and olive tree pruning (c).

When pretreatment was carried out at 210ºC, the condition for maximal sugar recovery in the liquid fraction, the solid fraction contained 9.7%, 13.6% and 8.0% of hemicelluosic sugars in ER, WS and OP, respectively, which corresponded to approximately 24-32% of their content in the raw material. Under the most severe condition (230ºC), the hemicellulose content in the solid fraction was residual (less than 3%). Accordingly, the pretreated solids of ER, WS and OP presented a significant higher glucan and Klason lignin contents at higher temperatures. Cellulose recovery under the conditions tested was above 95% for ER and WS and approx. 90% for OP, its content increasing in the solid fraction more than 20% when comparing autohydrolysis performed at 195ºC and 230ºC. These results are in agreement with previous reports for WS and corn stover (Buruiana et al., 2014; Carvalheiro et al., 2009). Moreover, this high fractionation of cellulosic (and lignin, as cellulo-lignin) and non-cellulosic sugars with autohydrolysis performed at 230ºC, might also be advantageous for further processing of cellulose by enzymatic hydrolysis, either for high glucan content and increased enzymatic digestibility (Yang et al., 2011).

2.3.2.3 Chemical post-hydrolysis of the liquid fraction

Chemical post-hydrolysis of the liquid fraction was optimized for maximal monosaccharide recovery. As OS are the main sugars present in the liquid fraction of pretreated materials, and in many cases those soluble sugars are still not suitable for biological or chemical conversion processes, a further conversion step is necessary for maximal monosaccharide recovery. Chemical post-hydrolysis, with dilute acid, is currently an interesting option (Duarte et al., 2004; Duarte et al., 2009; Garrote et al., 2001; Gírio et al., 2010) and, in this work, this process was optimized in order to identify the condition(s) for maximal sugar recovery, and minimal acid and energy (reaction time) consumption. The liquid fractions from autohydrolysis used for the optimization of the post-hydrolysis step were those obtained at 210ºC, which corresponded to highest sugar recovered (24.3, 40.2 and 38.2 g/L for ER, WS and OP, respectively) (Fig. 2.2).

In the post-hydrolysates, OS were hydrolyzed into monosaccharides reaching maximum yields at 1% sulfuric acid, 60 min, 121ºC (83, 90 and 93% for ER, WS and OP, respectively). In general, xylose was the most abundant monosaccharide found in the post-hydrolysates (11.6, 21.6 and 14.2 g/L for ER, WS and OP, respectively). Yet a significant glucose concentration was also observed in post-hydrolysates from WS and OP, which resulted from the high GlcOS and glucose (non-cellulosic glucan) content in the liquid fraction of the preceding autohydrolysis step with these two materials. Duarte et al., (2009) also found xylose as the predominant sugar for the post-hydrolysate of WS. Furans concentrations do increase with severity but remain close to the reported values for the autohydrolysis liquors (<2 g/L), except for the higher severities tested. Furthermore, furfural is the dominant furan for all feedstock (data not shown).

The regression coefficients of the proposed linear model for chemical post-hydrolysis of ER, WS and OP, together with the coefficient of determination (R^2) for the analyzed response, are presented in Table 2.2.

Table 2.2 Regression coefficients estimates[a] for the model of total sugars concentration from post-hydrolysis of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP).

	ER	WS	OP
β_0	20.86 ± 0.19 (0.00)	37.41±0.45 (0.00)	36.76±0.48 (0.00)
β_1	-0.21 ± 0.21 (0.35)	1.41±0.51 (0.03)	0.74±0.53 (0.21)
β_2	0.43 ± 0.21 (0.09)	1.89±0.51 (0.01)	0.95±0.53 (0.12)
β_{12}	-0.93 ± 0.21 (0.00)	-2.71±0.51 (0.00)	-1.84±0.53 (0.01)
R^2	0.80	0.89	0.74

[a] All values reported as coefficient ± standard error (*p*-value).

The acid concentration and reaction time were statistically significant effects in the recovery of total sugars from chemical post-hydrolysis of the liquid fraction of pretreated WS. Both had a positive effect for maximal sugar recovery. This result is in agreement with the maximal xylose recovery from XOS after chemical post-hydrolysis applied to the liquid fraction of pretreated WS obtained by Duarte et al., (2009).

The results of the chemical post-hydrolysis of the liquid fraction from autohydrolysis of ER and OP were different from those obtained from WS. In these cases, the main individual effects were not statistically significant in the confidence level defined ($p \leq 0.05$). However, the interaction coefficient for acid concentration and reaction time is statistically significant for total sugars recovery for the three materials studied. The negative effect on the studied response indicates that these variables act synergistically to increase sugar degradation.

In order to define the best conditions for the chemical post-hydrolysis process of liquid fraction generated during the autohydrolysis of ER, WS and OP, a constrained optimization model was implemented. Although other solutions were possible, the best conditions predicted by the models for maximal sugar recovery were obtained with low acid concentration (1% w/w) and longer isothermal reaction time (60 min) for all materials (Fig. 2.4). This condition corresponds to 4-fold reduction in acid consumption when compared to standard post-hydrolysis conditions (assay 1), using the same isothermal period. From an operational point of view, the implementation of such process with lower acid consumption (and alkalis for neutralization) will have also significant environmental and economic advantages.

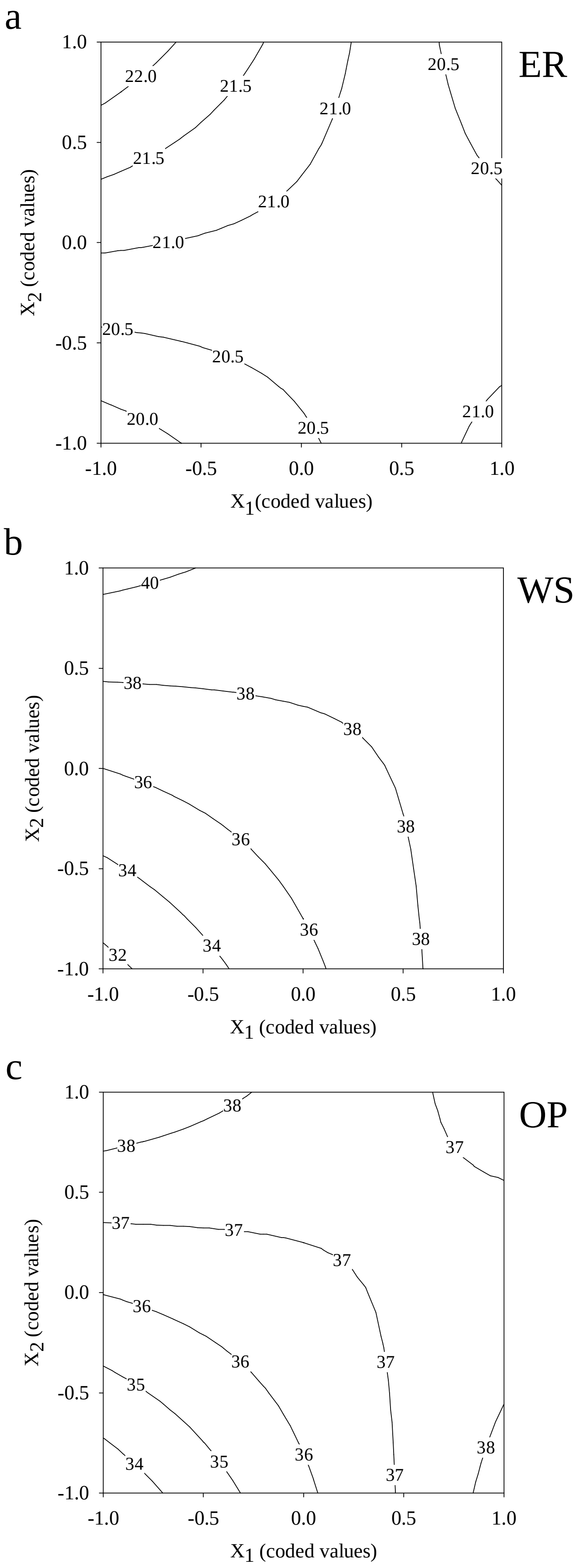


Figure 2.4 Control plot for total sugars recovery from post-hydrolysis of hemicellulosic hydrolysate from eucalyptus residues (a), wheat straw (b) and olive tree pruning (c) as a function of acid concentration (X_1) and reaction time (X_2).

2.3.2.4 Enzymatic hydrolysis of the solid fraction

The effect of autohydrolysis temperature on the efficiency of enzymatic hydrolysis of the solid fraction was assessed towards maximal glucose recovery. Two pretreatment temperatures were selected to evaluate enzymatic digestibility of the solid fraction resulting from autohydrolysis of ER, WS and OP: 210ºC, the condition providing maximal sugar recovery in the liquid fraction; 230ºC, the conditions providing the highest glucan content remaining in the solid fraction (Fig. 2.5). Under these conditions, i.e. autohydrolysis at 210 and 230ºC, the glucan content of the pretreated solids were, respectively: 49.5 and 52.4% for ER, 51.2 and 58.0% for WS and 40.5 and 42.1% for OP. Under the most severe conditions (230ºC), the solid fractions contained less than 3% xylan.

The conditions applied for enzymatic hydrolysis (5% (w/v) solid loading, 8 FPU/g_{solid} at pH 5.5, 50ºC) allowed a high rate of glucose release, reaching more than 80% of the final glucose titer after 12 h. After 48 h of enzymatic hydrolysis, the glucose yield obtained for the WS and OP pretreated solids at 230ºC was higher than 90%, whereas a lower yield (approx. 60%) was obtained for ER. The ER, WS and OP hydrolysates contained 17.1, 30.6 and 21.5 g/L of glucose, respectively.

The efficiency of enzymatic hydrolysis of the three materials pretreated at 210ºC was much lower than at 230ºC, reaching only 49.8, 78.8 and 64.8% of glucose yield for ER, WS and OP, respectively. The results revealed that a higher pretreatment temperature increased the yield of the enzymatic hydrolysis. Apart from higher physical disruption and consequent digestibility of cellulose fibers at higher temperature, the lower hemicellulose content in the solids reduces inhibition of cellulolytic enzymes (Alvira et al., 2010; Yang et al., 2011; Zhang et al., 2012). Furthermore, the composition and structure of each raw material and its combination with pretreatment temperature had also a significant impact on cellulose digestibility, as denoted by the different increase in glucose yields by enzymatic hydrolysis of solids obtained after autohydrolysis at 210 and 230ºC: increment of 9, 16 and 27% in glucose yield of ER, WS and OP. However, a higher enzyme dosage can contribute to increase enzymatic hydrolysis yield of ER (data not shown).

Previous reports of enzymatic hydrolysis of ER, WS and OP indicated yields of approx. 75% of glucose recovery, but pretreatment conditions, solids loading and enzyme dosage were differently combined (Cara et al., 2008b; Ertas et al., 2014; McIntosh et al., 2012).

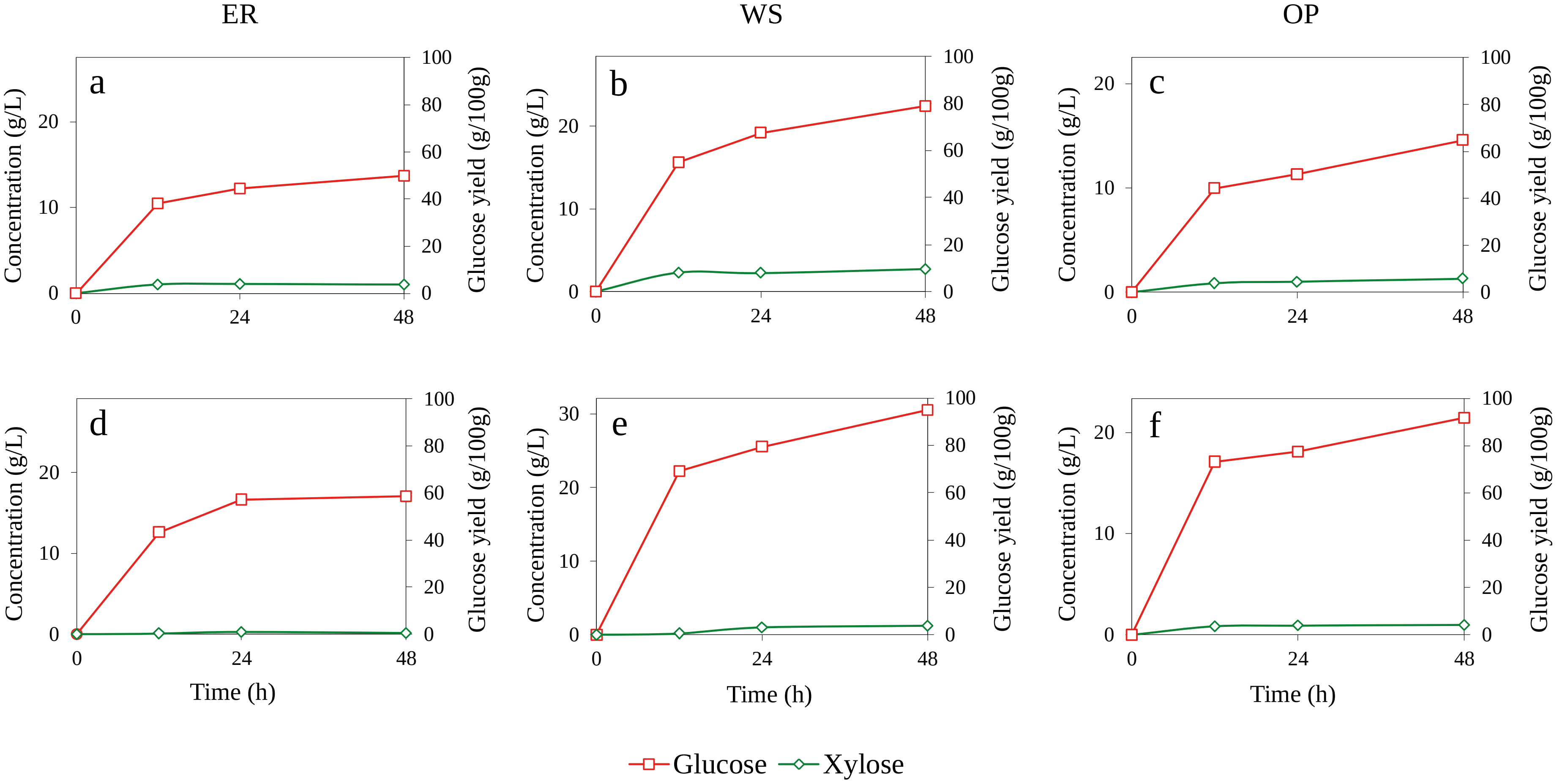


Figure 2.5 Kinetic profile of glucose and xylose concentrations and yield of enzymatic hydrolysis of the solids obtained after autohydrolysis, from eucalyptus residues (a, d), wheat straw (b, e) and olive tree pruning (c, f).

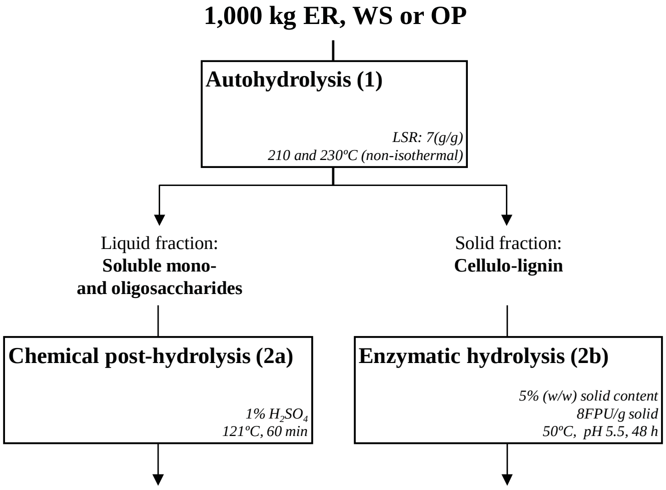
2.3.2.5 Overall sugar recovery

The maximal recovery of sugars is essential for the economy of biorefining processes based on biological conversion. Moreover, the fractionation processes based on hydrothermal or acidic pretreatment of lignocellulosic material are usually efficient on the recovery of the cellulose fraction in the solid and of the non-cellulosic sugars, as OS and monosaccharides, in the liquid fraction. Furans, organic acids and phenols, which are often inhibitors of biological conversion processes, are also solubilized or generated during the pretreatment and kept mainly in the liquid fraction. The purpose of this work was to evaluate the conditions for fractionation and maximal monosaccharides recovery from ER, WS an OP using three integrated biorefining processes: 1) autohydrolysis, 2a) chemical post-hydrolysis of its liquid fraction; 2b) enzymatic hydrolysis of its solid fraction as show the Fig. 2.6.

The yields of sugar recovery and the characterization of the two monosaccharides streams were compiled for two conditions of integrated biorefining processes: i) autohydrolysis at 210ºC, condition for maximal monosaccharide recovery after chemical post-hydrolysis (at 1% w/w sulfuric acid, 60 min, 121ºC) of the liquid fraction was obtained; ii) autohydrolysis at 230ºC, condition for maximal glucose recovery after enzymatic hydrolysis (5% (w/v) solids, 8 FPU/g_{solid} at pH 5.5, 50ºC) of the solid fraction was obtained.

In the integrated biorefining process, with the pretreatment at 210ºC, it was possible to obtain significantly higher recovery yields of monosaccharides in the liquid fraction (>70% of non-cellulosic sugars) when compared with the pretreatment at 230ºC. The very low recovery yield (<35%) of non-cellulosic monosaccharides with the pretreatment performed at 230ºC was due to significant higher generation of sugar degradation products mainly associated to the high severity of pretreatment. In opposite, the pretreatment at 230ºC favored the enzymatic hydrolysis of pretreated solids, with overall glucose recovery yields based on initial cellulose of 57.3% for ER, 97.2% for WS and 82.5% for OP, which represented a 18-25% higher glucose recovery in relation to the integrated process with pretreatment at 210ºC. Taking into account that a low enzyme dosage (8 FPU/g_{solid}) was used, there is still room for improvement of enzymatic hydrolysis, mainly with respect to the woody materials, like ER, where the maximum yields obtained were below 60% under the conditions tested.

The proposed biorefining process generated glucose-rich (cellulosic) streams (>89% of total monosaccharides) for all the materials and conditions tested, while the composition of the non-cellulosic stream (resulting from hemicellulose and non-structural sugars) was dependent of the lignocellulosic material (Fig. 2.6). Thus, while xylose represented about 50% of total monosaccharide content in non-cellulosic sugar streams from ER and WS, it was less than 40% of that from OP. In the case of the non-cellulosic sugar stream from OP, glucose content was similar or higher than xylose content.



	Monosaccharides (glucose/xylose)		Monosaccharides (glucose)		Total	
Lignocellulosic material	Autohydrolysis at 210ºC	Autohydrolysis at 230ºC	Autohydrolysis at 210ºC	Autohydrolysis at 230ºC	Autohydrolysis at 210ºC	Autohydrolysis at 230ºC
ER	161 (16/84)	66 (12/32)	210 (195)	232 (230)	371	298
WS	298 (74/161)	125 (41/60)	288 (257)	343 (330)	586	468
OP	284 (101/106)	111 (49/29)	173 (159)	235 (225)	457	346

Figure 2.6 Monosaccharides recovery (kg) from 1 ton eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP), after fractionation through autohydrolysis and enzymatic hydrolysis of the solid fraction and post-hydrolysis of the liquid fraction.

In sum, the results show that, although at 230ºC the pretreatments promoted higher enzymatic digestibility of cellulose, a significant amount of the sugars from the non-cellulosic fraction was degraded, becoming the pretreatment at milder conditions (210ºC) more attractive for total monosaccharide recovery from ER, WS and OP.

In this work, it was possible to generate 72.0% recovery of non-cellulosic monosaccharides for milder condition (autohydrolysis at 210ºC/post-hydrolysis) and 94.9% of enzymatic hydrolysis yield of the solid fraction for the most severe condition (autohydrolysis at 230ºC/enzymatic hydrolysis).

In sugar-based biorefineries, the integrated biorefining strategies should target a maximal sugar recovery and, at the same time, a fractionation process compatible with the subsequent biological or chemical conversion process. The bioconversion processes will depend on the capacity of microbial cell factories to convert the different monosaccharides present in lignocellulosic hydrolysates and also to tolerate the inhibitors generated during biomass deconstruction (sugar degradation products, phenolic compounds and organic acids). Therefore, different biorefining strategies to convert lignocellulose into fermentable sugars (monosaccharides) may be applied, making use of the different fractionation products obtained in this work. The residual solids after the enzymatic hydrolysis are rich in lignin, which might be suitable for lignin upgrading and commercialization, or for use in co-generation to produce energy and steam (CHP) to supply the energy demand of the biorefinery.

2.4 Conclusions

The integrated biorefining strategy presented here defines conditions for maximal monosaccharide recovery (pretreatment at 210ºC) and for maximal cellulose digestibility (pretreatment at 230ºC). The resulting monosaccharides are a sugar platform for the production of advanced biofuels, materials, bulk and fine chemicals by fermentation and/or chemical processes. The similar behaviour obtained for the different lignocellulosic materials opens the possibility of using a combination of these materials (mixtures) in order to minimize problems associated with feedstock availability and seasonality. The results obtained require validation at higher scale and solid content mainly with respect to pretreatment and enzymatic hydrolysis processes.

Acknowledgements

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Part B - Hydrothermal pretreatment of several lignocellulosic mixtures containing wheat straw and two hardwood residues available in Southern Europe

Abstract

Biomass availability and supply are key factors for the sustainability of biorefineries. The utilization of biomass mixtures is recognized as a solution to overcome these problems in certain regions, like Europe. This work studied the processing of biomass mixtures containing three lignocellulosic materials largely available in Southern Europe, eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP). The mixtures were chemically characterized, and their pretreatment, by autohydrolysis, evaluated within a severity factor (log R_0) ranging from 1.73 up to 4.24. A simple modeling strategy was used to optimize the autohydrolysis conditions based on the chemical characterization of the liquid fraction. The solid fraction was characterized to quantify the polysaccharide and lignin content. The best operational pretreatment condition for maximal saccharides recovery revealed to be at the same severity range (log R_0 3.65-3.72), independently of the mixtures tested, which suggests that autohydrolysis can effectively process mixtures of lignocellulosic materials.

Keywords

Biomass pretreatment; biorefinery, feedstock mixtures; lignocellulose; Southern Europe; biomass supply; sugar recovery

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Highlights

- Autohydrolysis was an efficient pretreatment for mixtures of lignocellulosic materials.
- Autohydrolysis profile is similar for different feedstock mixtures.
- The use of feedstock mixtures mitigate biomass supply constrains in biorefineries.
- Sugar-based biorefineries can use mixtures of hardwoods and agricultural residues.

Abbreviations

GlcOS, glucooligosaccharides; XOS, xylooligosaccharides; AOS, arabinooligosaccharides; HMF, 5-(hydroxymethyl)furfural.

2.6 Introduction

Many lignocellulosic residues, by-products and energy crops have been tested and laboratory and demonstration scales to be used as feedstock for biorefineries (Demirbas, 2009; Fava et al., 2013; FitzPatrick et al., 2010; Requejo et al., 2012; Saadatmand et al., 2012). However, the limited biomass availability and supply can be considered one of the major problems restraining the global biorefinery deployment (Balat, 2011). In fact, the seasonal nature and annual variability of biomass supply may turn the use of many feedstocks unpractical due to logistic/economic reasons (Yue et al., 2014), namely high transportation and storage costs. Moreover, feedstock flexibility and hence greater independence from suppliers is one of the key factors for making the feedstock cost stable during the lifetime of a biorefinery (Gnansounou, 2010). Typically, these barriers can be solved by the use of different biomass sources throughout the year, which may require special dedicated equipment and hence higher capital costs.

An alternative that can minimize the problems related with biomass availability, seasonality, and storage, is the use of biomass mixtures rather than a single raw material. Although some studies on biomass mixtures are already available (Jensen et al., 2008; Martin et al., 2008; Thomsen and Hauggaard-Nielsen, 2008), the focus on technical evaluation of processing feedstock mixtures in the initial biomass pretreatment/fractionation stage is still scarce.

In this work, eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) were chosen as representative feedstock from Southern Europe, as they are widespread and significantly available in relatively concentrated regions. For instances, their availability in Portugal is here estimated from available literature (Campilho, 2006; INE, 2012; INE, 2013; Kim and Dale, 2004; Romero-García, et al., 2014) to reached $1.1x10^6$ (ER), $7.6x10^4$ (WS), and $5.0x10^5$ (OP) ton per year. Furthermore, the seasonality of these materials is partially complementary, since ER is available throughout the year, WS is available in late spring/early summer, and OP is mainly available in winter and early spring. This further potentiates their use in a multi-feedstock biorefinery under continuous operation throughout the year, as also their storage costs are not expected to be significant as they have low moisture content, or can be easily dried naturally.

All these raw materials are polysaccharide-rich materials, reaching contents of 57% (ER), 68% (WS) and 62% (OP) of carbohydrate content. However, they present many differences, both at anatomical and chemical level. In fact, although they are all angiosperms, they have very different biological origins, belonging to significant different phylogenetic groups. Wheat is the classical example of an herbaceous crop (belonging to commelinids clade), eucalyptus is a typical hardwood and fast growing tree (belonging to the rosid clade), whereas olive tree is a slow-growing hardwood (belonging to the asterid clade). Their lignocellulose organization is significantly different, mainly with respect to hemicellulose and lignin composition. However, their hemicelluloses have the common trait of containing significant amounts of xylan.

As such, although it has already been proved that these materials can be successfully processed *per se* within the biorefinery framework (Chapter 2 – Part A; McIntosh et al., 2012; Negro et al., 2014; Pérez et al., 2008), their behavior in mixtures, specifically in the pretreatment/fractionation stage, is not known.

Hydrothermal treatments have already been demonstrated to be attractive pretreatment options for the biochemical upgrading of lignocellulosic materials. These processes require no other reagents than water (Carvalheiro et al., 2008) and have a clear marked nature of a self-catalyzed process, from where it is derived the name of autohydrolysis. In fact, these processes selectively hydrolyze the hemicellulose fraction and hence are prominently influenced by hemicellulose composition (Garrote et al., 2002; Ruiz et al., 2013), in contrast to the acid catalyzed processes. The latter is considered less environmental-friendly by the use of catalyst and neutralization agents. In fact, autohydrolysis promotes lower liberation of compounds derived from lignin and lower cellulose and hemicellulose degradation, which favor bioconversion processes, due to the lower levels of microbial inhibitors generated during pretreatment (Garrote et al., 2001). The remaining solid fraction from autohydrolysis is enriched in cellulose and sulfur-free lignin. Cellulose can be subjected to enzymatic hydrolysis to produce glucose, while the lignin can be recovered, e.g. as phenolic extracts (after delignification) or directly used for energy (co-)generation (Kang et al., 2013).

The main products found in the liquid fraction after autohydrolysis are oligosaccharides (OS), which cannot be directly metabolized by many microorganisms in bioconversion processes. Thus, a sequential hydrolysis process (post-hydrolysis step) can be applied to obtain a monosaccharides-stream to be used as sugar platform for the production of bio-based products. For this purpose, as compared to enzymatic post-hydrolysis, dilute-acid post-hydrolysis have competitive advantages, like the lower costs of catalysts and the shorter reaction time is required (Duarte et al., 2004; Duarte et al., 2009; Garrote et al., 2001) and the higher yield, the later mainly because hemicellulase commercial cocktails are still inefficient.

In this work, three combinations of ER, WS and OP were subjected to autohydrolysis at different severities (log R_0 from 1.73 up to 4.24) and its effect on the composition of liquid and solid fractions were evaluated. An empirical model was used as a tool to guide process optimization for each mixture and, at the higher OS content, a post-hydrolysis step of the liquid fraction was performed to evaluate maximal monosaccharides recovery.

2.7 Materials and Methods

2.7.1 Raw materials

ER are materials resulting from forest management practices, such as trimming and tree abatement, and were obtained from a commercial *Eucalyptus* farm (Mortágua, Portugal). They mainly consist of bark, branches and leaves and were provided already partially milled. WS was collected from an agricultural experimental station (Elvas, Portugal). OP was obtained from a local farmer (Ferreira do Alentejo, Portugal). Upon reception, ER and OP were dried at 40ºC to obtain moisture content less than 10% (w/w). All feedstock were then individually grounded with a knife mill to particles smaller than 6.0 mm, homogenized and stored separately in plastic containers at room temperature. These samples were used to prepare the different combinations of feedstock to be tested, as ER:WS:OP, equal 50:25:25; 25:50:25 and 25:25:50, also represented by 50ER, 50WS and 50OP, respectively. These combinations of feedstock were prepared as required, homogenized and then characterized as described below.

2.7.2 Autohydrolysis of different mixtures of eucalyptus residues, wheat straw and olive tree pruning

The autohydrolysis pretreatment was performed in a 0.6-L stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature controller (model 4842). Each mixture (40 g dry basis) was loaded with water to obtain a liquid-to-solid ratio of 7 ($g.g^{-1}$) and the agitation speed was set at 150 rpm. For each mixture, ten different final temperatures in the range of 150°C to 230°C were tested, under non-isothermal conditions, so that when the desired temperature was reached, the reactor was immediately cooled down.

Liquid and solid fractions were separated using a hydraulic press (Sotel, Portugal) up to 200 kg/cm^2 and the liquid fraction recovered was filtered (Whatman no. 41 filter paper) to remove the remaining solids. The solid fraction was washed at room temperature with two volumes of water, filtered and dried at 40°C before analysis of the chemical composition.

The effects of time and temperature on autohydrolysis were interpreted based on the severity factor, log R_0 (Overend and Chornet, 1987):

$$R_0 = \int_0^t \exp\left(\frac{T(t) - 100}{14.75}\right) dt$$

where t is time (min), T the temperature (°C), and 14.75 an empirical parameter related with activation energy.

2.7.3 Empirical modelling

The experimental data were fitted iteratively to empirical polynomial models implemented in MSExcel© 2010. The determination of the best operational conditions maximizing saccharides recovery was obtained using the Solver function, also built-in in MSExcel© 2010, based on 4th order polynomial models, as shown in the Eq. 1.

$$Y = \beta_4 x^4 + \beta_3 x^3 + \beta_2 x^2 + \beta_1 x + \beta_0 \text{ (Eq.1)}$$

where, Y is the yield of the studied variables (g/100 g feedstock mixture), β_0, β_1, β_2, β_3 and β_4 are coefficients of the polynomial and x is the severity factor.

The studied variables were the yields of GlcOS, XOS, AOS and total OS, their corresponding monomers as glucose, xylose and arabinose, and also sum of pentoses and total saccharides (oligo- and monosaccharides), acetic and formic acids, sugars degradation products as furfural and HMF and total phenolic compounds.

2.7.4 Chemical post-hydrolysis of the liquid fraction from autohydrolysis

The liquid fractions recovered from autohydrolysis were subjected to a second hydrolysis process under previously optimized chemical post-hydrolysis conditions for maximal sugar recovery, i.e. using sulfuric acid, 1% (w/w) final concentration, at 121ºC for 60 min (Chapter 2 – Part A).

2.7.5 Analytical Methods

2.7.5.1 Chemical characterization of the solid fraction

Raw materials, as mixtures (50ER, 50WS and 50OP), and respective pretreated solids were characterized by quantitative acid hydrolysis followed by HPLC analysis, after being ground to particles smaller than 0.5 mm. The samples were mixed with 72% (w/w) sulfuric acid for 60 min at 30ºC, diluted with water to 4% (w/w) sulfuric acid and hydrolyzed for 60 min at 121ºC. The solids obtained were used to determine the acid insoluble lignin (Klason lignin) content, after correction for ash. Ash content was determined by igniting the contents at 550ºC for 5 h. The hydrolysate from quantitative acid hydrolysis was analyzed by HPLC (Agilent 1100 Series, Germany), using an Aminex HPX-87H column (Bio-Rad, Hercules, CA), operating at 50°C. Monosaccharides (glucose, xylose and arabinose) and acetic acid were eluted, after injection of a 20 μL sample, with sulfuric acid (5 mM) as mobile phase at 0.4 mL/min flow rate.

The soluble saccharides content of the three mixtures (*in natura*) was evaluated by extraction using the National Renewable Energy Laboratory methodology (TP-510-42619) and quantification by HPLC. Protein content was estimated by the Kjeldahl method, using the N x 6.25 conversion factor and fat was quantified using ethyl ether according to Portuguese standard 876. All the analyses were performed in triplicate. Total phenolic compounds content was assayed spectrophotometrically at 765 nm by Folin-Ciocalteu method (Singleton et al., 1999) using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific, MA, USA). Gallic acid was used as calibration standard.

2.7.5.2 Chemical characterization of liquid fraction from autohydrolysis and acid post-hydrolysis

Filtered samples of the liquid fractions from autohydrolysis and post-hydrolysis were analyzed directly by HPLC, under the conditions described above, except for flow rate and injection volume, which were set at 0.6 mL/min and 5 μL, respectively. OS were measured by an indirect method based on quantitative acid hydrolysis of the liquid fraction as previously described (Carvalheiro et al., 2004). OS concentrations were calculated from the increase of the respective sugar monomers, after acid post-hydrolysis.

2.8 Results and Discussion

2.8.1 Chemical characterization of different mixtures of eucalyptus residues, wheat straw and olive tree pruning

Three mixtures with different combinations of ER, WS and OP were used in this study. In each mixture a single feedstock was set at 50% of the total weight and the other two contributed with 25% each. Thus, these three ternary mixtures were represented as 50ER, 50WS and 50OP. These mixtures aimed to model possible feedstock mixtures that can be available throughout the year, corresponding to the larger availability of each raw material according to their seasonality.

In general all feedstock mixtures studied revealed a very similar chemical composition (Table 2.3), although the content of each fraction slightly vary, according to the contribution of the predominant feedstock. Glucan was the polysaccharide found in higher amount and its values varied between 30.0 and 32.7%, with the highest content found in 50ER, in agreement with the higher glucan content of ER (Chapter 1 – Part A). The glucan content (cellulose and starch) for individual materials was accounted for 36.1, 33.7 and 26.8% for ER, WS and OP, respectively (Chapter 1 – Part A). The glucan content determined for each mixture was less than 5% different than the weighted average of the obtained for each individual material. Similarly, among the hemicellulose components, the xylan was found in the highest amount in all mixtures, reaching 19.2% in 50WS, which is in agreement with the higher xylan content of WS (Chapter 1 – Part A). Galactan and mannan were detected in residual amounts in each of the feedstock used (Chapter 1 – Part A) and therefore were neglected in the study of mixtures. Arabinan was identified but present in low proportions in all mixtures (<3%). The acetyl groups content slight varied between the mixtures and, as expected, it has been found in higher proportions in the 50ER mixture, which is due the hemicellulose nature of ER, more acetylated than WS and OP (Cara et al., 2008; Chapter 1 – Part A; Pérez et al., 2008). Klason lignin constituted a significant fraction of the mixed materials, and their content corresponded also to the weighted average of the lignin found in individual materials (26.7, 16.8 and 22.2, for ER, WS and OP, respectively), with less than 2% difference. In fact, the mixture containing higher amount of WS (50WS) revealed the lowest lignin content (20.6%), which is in agreement with the characteristic low lignin abundance in this agriculture residue.

Table 2.3 Average macromolecular composition of three different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP).

Component	Feedstock mixture		
	50ER	50WS	50OP
Glucan	32.7 ± 0.4	31.7 ± 0.0	30.0 ± 0.2
Xylan	17.9 ± 0.4	19.2 ± 0.1	17.4 ± 0.2
Arabinan	2.2 ± 0.1	2.7 ± 0.1	2.8 ± 0.1
Acetyl groups	4.2 ± 0.6	3.6 ± 0.4	4.0 ± 0.6
Klason lignin	23.5 ± 0.3	20.6 ± 0.1	22.0 ± 0.1
Ash	3.9 ± 0.0	4.5 ± 0.1	4.3 ± 0.1
Protein	3.6 ± 0.1	4.2 ± 0.1	4.5 ± 0.0
Fat	2.5 ± 0.1	2.5 ± 0.1	3.2 ± 0.4
Soluble saccharides	4.5 ± 0.5	5.2 ± 0.3	6.4 ± 0.4

Others constituents such as ash, protein, fat and soluble saccharides were quantified. Ash, protein or fat content varied less than 1% between mixtures. Instead, the soluble saccharides content of the three mixtures reflects the very different nature of the individual materials. As expected, the mixture with higher proportion of OP presented the highest soluble saccharides content, as OP has been reported to contain 12.0%, while WS and ER, 5.3 and 1.7%, respectively (Chapter 1 – Part A).

2.8.2 Product recovery in the liquid fraction and modeling

The autohydrolysis process was carried out for the tested mixtures of 50ER, 50WS and 50OP, under non-isothermal conditions (150-230ºC), corresponding to a severity factor (Log R_0) between 1.73 and 4.24. The severity factor is an empirical parameter that combines the effect of relevant operational variables, i.e., temperature and time (Overend and Chornet, 1987) and enables an easier comparison among different tested conditions. For a given final temperature the severity factor determined was slightly different for each mixture since besides the specific time/temperature profile, appeared to be also influenced by the intrinsic features of the lignocellulosic material.

After pretreatment, the data obtained for the products recovered in the liquid fraction were interpreted using an empirical model based on a polynomial equation. Empirical models can provide a mathematical description of processes and can be used iteratively, since are especially helpful to identify the data points required to better define the experimental domain and to fine-tune the best operational conditions for a given goal (e.g. maximum yields). Thus, in order to model product recovery, 4th order polynomial models were tested as these were the one with the lower order found to better capture the system behavior. The fitting between experimental data and the adjustments obtained by the models (in most cases R^2 >0.90) showed that these models seem to adequately represent the autohydrolysis profile.

The products recovered in the liquid fraction after autohydrolysis of 50ER, 50WS and 50OP at different severities were represented in three groups (Fig. 2.7): OS, including XOS, AOS and GlcOS (Fig. 2.7a-c); monosaccharides, including xylose, arabinose and glucose (Fig. 2.7d-f); and other by-products such as organic acids (acetic and formic), furans (furfural and HMF) and total phenolic compounds (Fig. 2.7g-i).

The recovery yields of the main compounds were modeled to fine-tune the operational conditions leading to the maximum recovery of saccharides. The equations for the recovery of OS and monosaccharides (in g/100g feedstock mixture) were determined (Table 2.4).

In general, a similar profile of OS recovery was observed for all the mixtures studied. As expected, XOS were the main OS recovered in all feedstock mixtures. The highest XOS recovery obtained for 50ER, 50WS and 50OP were 11.3, 12.0 and 11.8 g/100g feedstock mixture, respectively, equivalent to 63-68 g/100 g of original xylan. These results were obtained for severity factor between 3.62 and 3.75. The model further narrowed the range for maximum OS recovery at severity factors between 3.69 and 3.73. The values obtained for XOS recovery decreased considerably at more severe conditions to reach 1.9-3.0g/100 g feedstock mixture at severity of 4.2. Under this condition xylan solubilization reached 71-75%. AOS were detected in the liquid fraction due the solubilization of arabinan, and as XOS, were degraded for higher severities. The maximal value found for AOS recovery was that predicted by the model (2.4g/100g), but at lower severity than XOS. GlcOS were also detected in the liquid fraction and their maximum recoveries were found at the same severities than those of XOS. Their production is mainly attributed to the presence of non-structural glucan, such starch and soluble saccharides (Cara et al., 2012; Chapter 1 – Part A). Therefore, at the lowest pretreatment severity tested, GlcOS were already recovered at 1.4, 1.7 and 2.0 g/100g for 50ER, 50WS and 50OP, respectively, while the maximum was estimated in 3.3, 4.2 and 5.2 g/100g and corresponded to a severity factor of 3.43, 3.59 and 3.47, respectively.

Xylose recovery yield continuously increased with pretreatment severity, since harsher conditions promote XOS hydrolysis. However, the maximum arabinose recovery yield was obtained under less severe conditions than those found for xylose. This observation is consistent with the more labile nature of arabinose and their presence in ramifications of hemicellulose rather than in the backbone. For the mixture 50ER pentose recovery yield increased up to a severity factor of 3.72, reaching a value of 4.6 g/100g feedstock mixture. The maximum pentose yield observed for 50WS was 4.6 g/100g feedstock mixture, at a severity range between 3.77-4.24. For the mixture 50OP a maximum pentose yield of 5.1 g/100g was obtained at 3.91 severity factor.

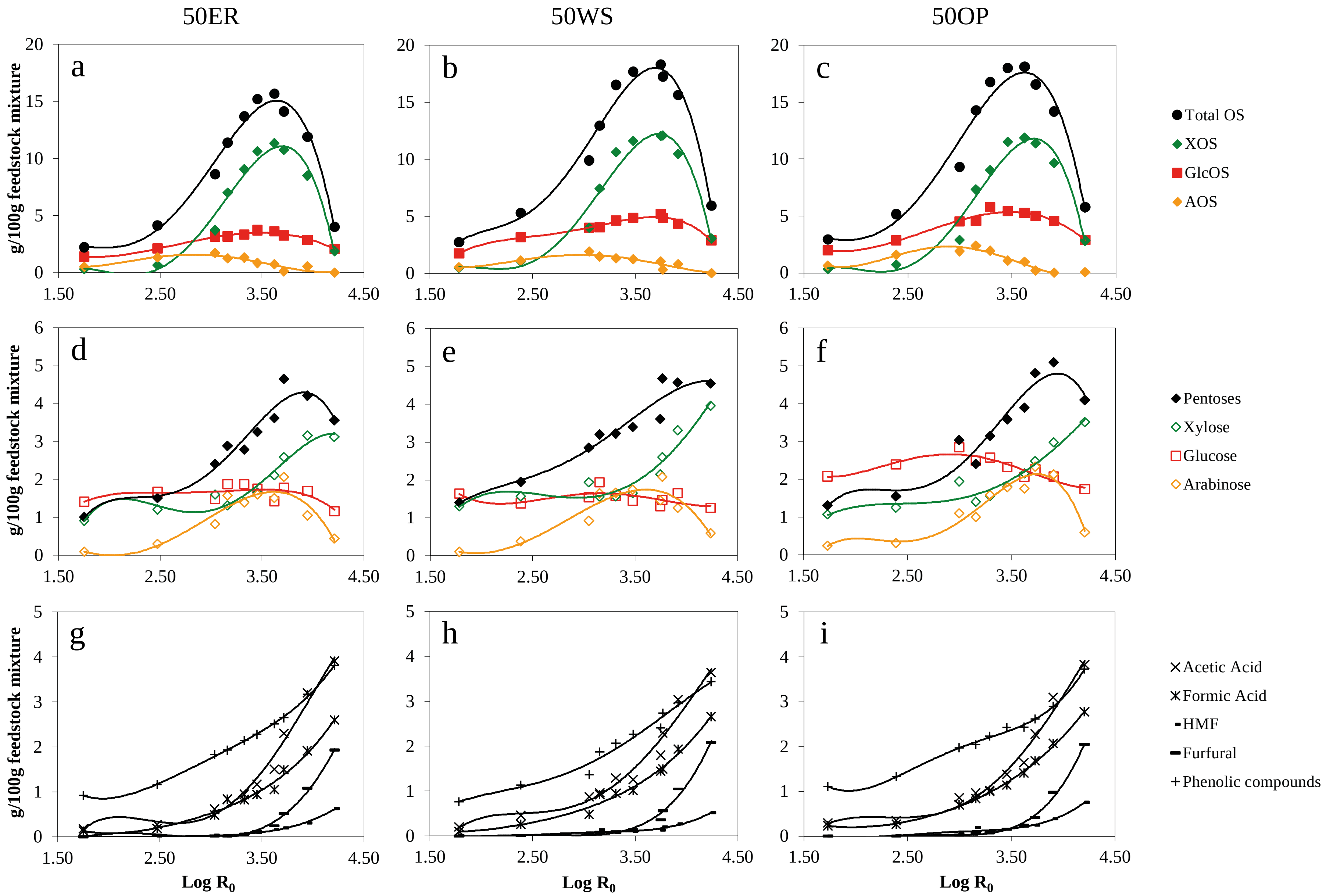

Figure 2.7 Fitted models based on 4th order polynomials adjusted for yield of oligosaccharides, monosaccharides, organic acids, furans and total phenolic compounds after autohydrolysis of the three different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) as a function of the pretreatment severity.

Table 2.4 Polynomial models for oligosaccharides and monosaccharides recovery yield from three different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) and the respective coefficient of multiple determination (R^2).

Feedstock mixture	Polynomial Models	R^2
50ER	XOS = $-4.19x^4 + 42.70x^3 - 154.24x^2 + 237.08x - 131.68$ (2)	0.98
	AOS = $0.60x^4 - 6.85x^3 + 27.80x^2 - 46.72x + 28.27$ (3)	0.85
	GlcOS = $0.05x^4 - 1.50x^3 + 9.71x^2 - 21.74x + 17.28$ (4)	0.98
	Total OS = $-3.54x^4 + 34.34x^3 - 116.74x^2 + 168.62x - 86.13$ (5)	0.98
	Xylose = $-0.89x^4 + 10.92x^3 - 48.12x^2 + 90.91x - 60.99$ (6)	0.95
	Arabinose = $-0.25x^4 + 1.95x^3 - 4.44x^2 + 2.15x + 1.79$ (7)	0.88
	Glucose = $-0.26x^4 + 3.01x^3 - 12.72x^2 + 23.65x - 14.67$ (8)	0.57
	Pentoses = $-1.14x^4 + 12.88x^3 - 52.57x^2 + 93.06x - 59.20$ (9)	0.95
	Total saccharides = $-4.95x^4 + 50.22x^3 - 182.02x^2 + 285.33x - 159.99$ (10)	0.99
50WS	XOS = $-4.27x^4 + 44.09x^3 - 162.05x^2 + 254.92x - 145.56$ (11)	0.96
	AOS = $0.42x^4 - 5.10x^3 + 21.83x^2 - 38.44x + 24.32$ (12)	0.84
	GlcOS = $-1.16x^4 + 13.02x^3 - 54.10x^2 + 99.69x - 66.14$ (13)	0.98
	Total OS = $-4.96x^4 + 51.52x^3 - 192.29x^2 + 312.55x - 185.03$ (14)	0.98
	Xylose = $-0.23x^4 + 3.38x^3 - 17.10x^2 + 35.93x - 25.30$ (15)	0.92
	Arabinose = $-0.12x^4 + 0.64x^3 + 0.58x^2 - 5.88x + 6.37$ (16)	0.88
	Glucose = $0.28x^4 - 3.52x^3 + 16.31x^2 - 32.31x + 24.58$ (17)	0.38
	Pentoses = $-0.35x^4 + 4.03x^3 - 16.52x^2 + 30.05x - 18.93$ (18)	0.94
	Total saccharides = $-5.04x^4 + 52.02x^3 - 192.50x^2 + 310.29x - 179.38$ (19)	0.99
50OP	XOS = $-4.82x^4 + 50.00x^3 - 185.28x^2 + 293.84x - 169.18$ (20)	0.96
	AOS = $1.54x^4 - 18.11x^3 + 76.23x^2 - 135.42x + 86.77$ (21)	0.94
	GlcOS = $0.03x^4 - 1.92x^3 + 14.03x^2 - 33.04x + 26.83$ (22)	0.96
	Total OS = $-3.24x^4 + 29.97x^3 - 95.03x^2 + 125.38x - 55.57$ (23)	0.96
	Xylose = $-0.14x^4 + 1.97x^3 - 9.49x^2 + 19.22x - 12.76$ (24)	0.94
	Arabinose = $-1.08x^4 + 11.76x^3 - 46.39x^2 + 78.97x - 48.79$ (25)	0.95
	Glucose = $0.30x^4 - 3.55x^3 + 14.90x^2 - 25.92x + 18.03$ (26)	0.86
	Pentoses = $-1.21x^4 + 13.73x^3 - 55.88x^2 + 98.19x - 61.55$ (27)	0.93
	Total saccharides = $-4.16x^4 + 40.14x^3 - 136.01x^2 + 197.65x - 99.09$ (28)	0.99

Values of the maximum pentose recovery predicted by the models were observed for the severity factors of 3.92, 4.24 and 4.03 for 50ER, 50WS and 50OP, respectively. Glucose yield was lower than 3.0 g/100g for all mixtures, being relatively constant at all the severity conditions tested.

The maximum saccharides recoveries experimentally obtained in the liquid fractions for 50ER, 50WS and 50OP were 20.7, 23.4 and 24.0 g/100 g feedstock mixture, respectively. The respective recoveries predicted by the models (eq. 10, 19 and 28) were less than 5% different, specifically 20.0, 23.8 and 23.9 g/100 g feedstock mixture for 50ER, 50WS and 50OP, respectively, corresponding to severity factor of 3.67 (50ER), 3.72 (50WS) and 3.65 (50OP).

Furans, organic acids and total phenolic compounds yields displayed a similar profile in the liquid fractions of pretreated 50ER, 50WS and 50OP. Furfural, a degradation product from pentoses, increased with pretreatment severity reaching values in the order of 2 g/100g under the most severe conditions. HMF, a degradation product from hexoses, was also found in higher amounts at the most severe conditions, reaching 0.52-0.75 g/100g feedstock mixture. Similar to the observed for furans, the higher acetic and formic acids and total phenolic compounds yields were obtained under the most severe conditions. The organic acids resulting from acetyl groups hydrolysis (acetic acid) and from furfural and HMF break down (formic acid) (Palmqvist and Hahn-Hägerdal, 2000), reached values lower than 4 and 3 g/100g feedstock mixture, respectively. Total phenolics, the compounds resulting from degradation product of lignin (Palmqvist and Hahn-Hägerdal, 2000), reached recovery yields between approximately 1.0 to 3.4-3.8 g/100 g feedstock mixture, under the least and the most severe conditions, respectively.

2.8.3 Characterization of the liquid fraction recovery after autohydrolysis and chemical post-hydrolysis

For maximum recovery of total saccharides in the liquid fraction, the model predicted severity factor in the range of 3.65-3.72, which corresponded to a pretreatment temperature around 210ºC. The composition of the liquid fraction of 50ER, 50WS and 50OP pretreated at this temperature under non-isothermal condition (severity factor of 3.62-3.75), is shown in Table 2.5. The different liquid fractions presented similar composition, although some products were present in slightly higher amounts, as a function of the predominance of each raw material.

XOS were the main component of the liquid fractions, which, under the conditions applied (liquid:solid ratio of 7:1), reached concentrations of 15-16 g/L (14.6-16.6 g/L, as predicted by the models) and revealed that is possible to achieve consistent product concentration regardless of the mixture used. GlcOS were the second most abundant OS in the liquid fraction, reaching 4.9-7.1 g/L. Values between 4.3 and 6.9 g/L were predicted by the models for the conditions of maximal saccharides recovery. On the other hand, AOS were found at low concentration (<1.5 g/L). For AOS recovery, values slight higher were predicted by the models (1.3-2.0 g/L).

Under the same conditions each monosaccharide was obtained at low concentrations (3 g/L), since autohydrolysis solubilize the hemicellulose fraction manly in oligomeric form (Carvalheiro et al., 2008). Acetic acid was found at slightly higher concentrations than formic acid (Table 2.5), suggesting low degradation of hemicellulosic sugars. In fact, the concentrations of other sugars degradation products, such as furfural and HMF, were also low. Total phenolic compounds were present in very similar concentrations for all feedstock mixtures (approx. 3.3 g/L).

Table 2.5 Composition (g/L) of the liquid fraction obtained after autohydrolysis of the three different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) in the experimental condition of the maximal saccharides recovery.

Component	Feedstock mixture		
	50ER	50WS	50OP
GlcOS	4.92	7.03	7.10
XOS	15.37	16.28	15.91
AOS	0.98	1.40	1.29
Glucose	1.93	1.77	2.76
Xylose	2.86	2.90	2.89
Arabinose	2.05	1.98	2.34
Acetic acid	2.04	2.43	2.20
Formic acid	1.43	1.95	1.89
HMF	0.22	0.17	0.29
Furfural	0.33	0.49	0.34
Total phenolic compounds	3.41	3.34	3.26

The liquid fractions resulting from autohydrolysis (at 210ºC) of feedstock mixtures were subject to chemical post-hydrolysis in order to quantify the maximum monosaccharides recovery. As expected, for all feedstock mixtures, xylose was the monosaccharide obtaining at higher concentrations (17.4-20.4 g/L), followed by glucose (7.0-9.9 g/L) and arabinose (2.5-3.0 g/L). Under these conditions of post-hydrolysis, pentoses account for about 68-74% of all monosaccharides. Furfural and HMF were obtained in higher concentrations than those quantified in the liquid fraction obtained after autohydrolysis. This fact is explained by the degradation of monosaccharides present in the liquid fraction from autohydrolysis. Furfural was obtained at higher concentration than HMF, reaching 1.5-2.0 g/L, since the pentoses are more vulnerable to thermal degradation than hexoses. Formic and acetic acids also increased up to 1.6 and 4.5 g/L, respectively. This has been attributed to furan degradation and to extensive hydrolysis of hemicellulose oligomers, respectively. Levulinic acid was only detected after chemical post-hydrolysis, and at similar concentration (<0.5 g/L) for all feedstock mixtures studied, most probably resulting from degradation of HMF (Palmqvist and Hahn-Hägerdal, 2000).

The sugar components of these post-hydrolysates can be upgraded by biological or chemical conversion processes for the production of biofuels, biochemicals or biomaterials. The biological conversion of such materials may be hampered by the presence of microbial inhibitors, like acetic acid, furfural and phenolic compounds. Yet, autohydrolysis and mild acid post-hydrolysis generate liquid fractions with relatively low inhibitory compounds, when compared to those obtained by other pretreatment methods, like dilute-acid pretreatment. Biological conversion processes will also benefit from the integrated use of all fractionated sugars, combining the use of pentoses from hemicellulose with cellulosic and non-structural glucose.

2.8.4 Characterization of the solid fraction

During autohydrolysis, the extension of hemicellulose solubilisation is directly proportional to autohydrolysis severity, while cellulose and lignin are usually retained in the solid fraction (Carvalheiro et al., 2009). In fact, a decrease of hemicellulosic components (xylan, arabinan and acetyl groups) in the solid fraction was observed with the increase of pretreatment severity for all feedstock mixtures, demonstrating the fitness of these substrates to be efficiently fractionated by autohydrolysis (Fig. 2.8). Arabinan and acetyl groups were removed from the solid fraction under mild pretreatment conditions. Under the most severe conditions (severity factor of 4.2), the xylan was extensively removed from the solids (93-95% of solubilisation), regardless of the mixtures.

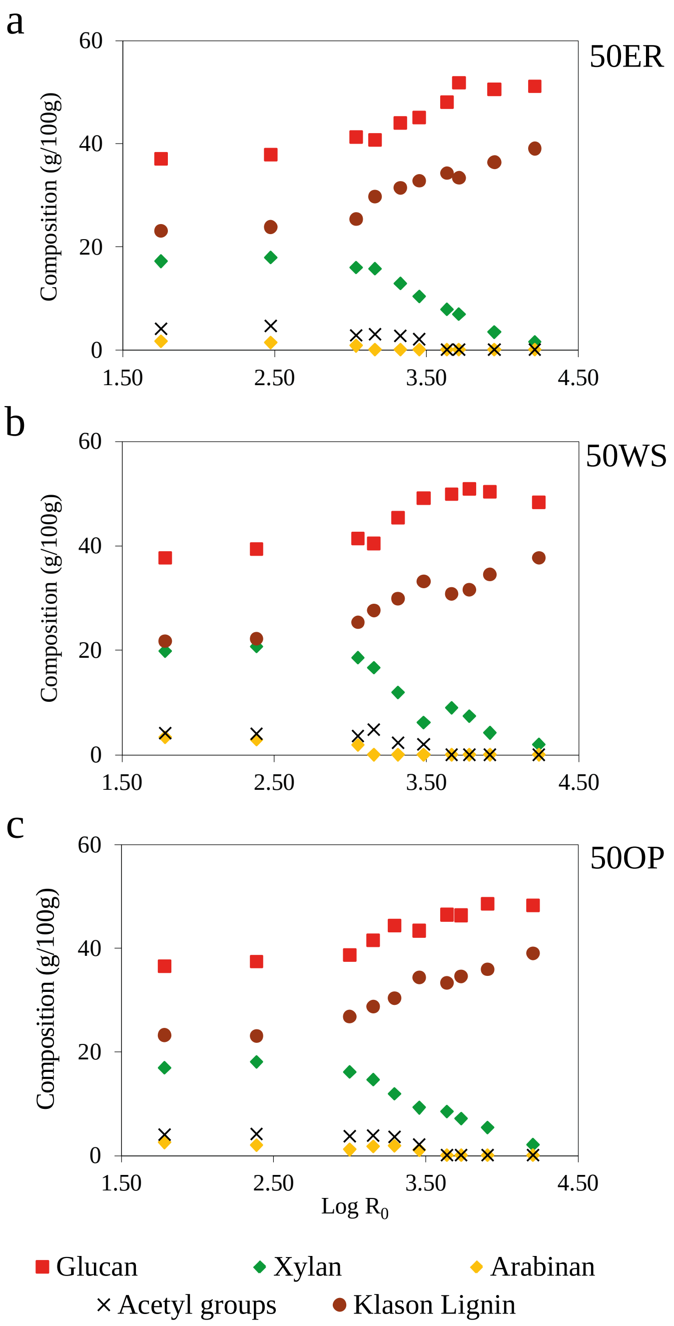

Figure 2.8 Effect of pretreatment severity in the composition of the solid fraction recovered after autohydrolysis of the three different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP).

Furthermore, an increase in both glucan and Klason lignin content was observed with the increase of severity factor, showing the low solubilisation of these components. In fact, glucan solubilisation does not exceed 10% and lignin is virtually kept entirely in the solid fractions under all tested conditions. Under the most severe conditions, the solid fraction of 50ER, 50WS and 50OP contained 51.1, 48.3 and 48.3% of glucan and 39.0, 37.7 and 39.0% of lignin, respectively, i.e. these two components represent more than 85% of the solid fraction, regardless the mixture. The relative proportions of these two components were maintained in relation to their native composition in mixtures.

This cellulose and lignin can be upgraded. The cellulose usually undergoes enzymatic hydrolysis before biological conversion into biofuels, biochemicals or biomaterials, as single substrate or in combination with sugars obtained from the liquid fraction of autohydrolysis (and subsequent post-hydrolysis), as described above. Lignin can be upgraded to phenolic extracts and biomaterials or directly used for energy (co-)generation (CHP) in the framework of a biorefinery (Carvalheiro et al., 2008; Kang et al., 2013). The uses of the cellulosic and lignin fractions recovered after autohydrolysis of lignocellulosic mixtures should be further investigated for integrate valorization, but it is out of the scope of the current work.

2.9 Conclusions

This work demonstrated that mixtures of lignocellulosic materials can be efficiently processed by autohydrolysis, under similar conditions, and generate consistent product composition independently of the different proportions of each feedstock. The three mixtures of ER, WS and OP are particularly attractive for a multi-feedstock biorefinery in Southern Europe operating throughout the year at constant operational conditions for the pretreatment step. The viability of such biorefinery requires further testing at the level of enzymatic hydrolysis of pretreated solids and bioconversion of both the cellulosic and hemicellulosic hydrolysates.

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CHAPTER

3

ENZYMATIC HYDROLYSIS OF HYDROTHERMAL PRETREATED LIGNOCELLULOSIC MATERIALS

Optimization of glucose recovery by enzymatic hydrolysis of hydrothermally pretreated eucalyptus residues, wheat straw or olive tree pruning and their mixtures

Abstract

Eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) are lignocellulosic materials largely available in Southern Europe and, due to their high polysaccharide content, have high potential to be used in biorefineries for the production of fuels, chemicals and materials through biochemical routes. For this, is crucial to have an efficient enzymatic hydrolysis of cellulose. As the key factors determining the efficiency of cellulose enzymatic hydrolysis are: the severity of the pretreatment applied to the feedstock, the enzyme dosage and solids loading applied in the process. The aim of this work was to study these three variables in the enzymatic hydrolysis of ER, WS, OP and their mixtures, towards the optimization of glucose recovery. The effect of the temperature of hydrothermal pretreatment (non-isothermal autohydrolysis at 210-230°C), enzyme dosage (0.25-0.75 g_{enzyme}/g_{solids} of a Celluclast® 1.5L/Novozyme® 188 cocktail) and solids loading (5-20% w/v) was studied using a 2^3 complete factorial central composite design followed by response surface methodology analysis. For each feedstock, statistical models were developedand used to identify the conditions giving the maximization of glucose concentration and yield. Under the conditions tested, the model was able to predict conditions providing enzymatic hydrolysis yields above 85% for each feedstock. Moreover, the results allowed to define optimal conditions for the enzymatic hydrolysis of different feedstock mixtures (ER:WS:OP) at 20% (w/v) solids, maintaining yields of glucose recovery over 85%. The optimal conditions were further experimentally validated.

Keywords

Biorefinery; enzymatic hydrolysis; feedstock mixtures; lignocelluosic materials; response surface methodology; sugar platform.

Highlights

- Results of enzymatic hydrolysis of individual feedstock were used to predict those of feedstock mixtures.
- The optimal enzymatic hydrolysis conditions at 20% (w/v) pretreated solids are similar for all feedstock mixtures.
- Similar enzymatic hydrolysis conditions, at 20% (w/v) pretreated solids, can be applied for high glucose recovery from all feedstock mixtures.
- From 20% (w/v) pretreated solids, more than 85 g/L glucose (>80% yield) were obtained for individual feedstock their mixtures.

Abbreviations

DF, degrees of freedom; pretreatment temperature (X_1); enzyme dosage (X_2) and solid loading (X_3)

3.1 Introduction

Lignocellulosic materials are renewable and abundant biomass resources and, due to their generally high polysaccharide content, constitute a potential source of fermentable sugars. These sugars can be converted into countless bio-based products, including biofuels, biochemicals and biomaterials, through chemical or biological processes (FitzPatrick et al., 2010; Ghatak, 2011). ER, WS and OP are lignocellulosic materials largely available in Southern Europe, and their use in sugar-based biorefineries is attractive due to their high saccharide content (57-68%) (Chapter 2 – Part A). The use of mixtures containing these lignocellulosic materials (ER:WS:OP) can bring additional benefits in the operation of biorefineries while minimizing problems related with feedstock availability and supply, including seasonality and storage constrains, which can arise when a single raw material is used (Chapter 2 – Part B).

The deconstruction of lignocellulosic materials is considered the key stage for the success of a biorefinery based on biochemical routes, due to the impact of this step on the recovery of fermentable sugars from the polysaccharides (Sun and Cheng, 2002). The major barrier to the success of this step is the recalcitrance of lignocellulosic materials, which requires physical, chemical and/or biological pretreatment to initiate the deconstruction of the rigid structure created by their three major components: cellulose, hemicellulose and lignin (Hatakka, 1983; Overend and Chornet, 1987; Taniguchi et al., 1982). Several pretreatment methodologies have been proposed, with many of them utilizing chemical catalysts such as acid, alkaline, ionic liquids, solvents (Alvira et al., 2010; Carvalheiro et al., 2008; Taherzadeh and Karimi, 2008). The hydrothermal (self-catalysed) pretreatment has proven to be one of the most attractive processes, with several advantages, like the use of water as the only external catalyst, the generation of low sugars degradation products, the limited problems derived from equipment corrosion, owing to the mild pH of reaction media, and the relatively low operational costs since further neutralization can be omitted (Gírio et al., 2010; Ruiz et al., 2013). Under this pretreatment the polysaccharides from hemicellulosic fraction are hydrolysed, more or less extensively depending on the severity conditions (e.g. temperature), resulting in a liquid fraction rich in oligosaccharides, while, in the solid fraction, the cellulose crystallinity is reduced and the porosity of the material (cellulo-lignin) is increased, facilitating the subsequent enzymatic hydrolysis of the cellulosic fraction (Garrote et al., 1999).

The hydrolysis of the cellulosic fraction can be performed through chemical or enzymatic processes (Balat, 2011; Sanchez and Cardona, 2008), but the later has been considered the most interesting for the majority of commercial and pre-commercial sugar-based biorefineries. Although the enzymes required for the complete hydrolysis of cellulose still represent one of the major operational costs of these biorefineries (Modenbach and Nokes, 2013), there are several advantages that make enzymatic hydrolysis an attractive process over chemical hydrolysis, such as lower equipment costs, mild operating conditions (pH and temperature), no requirement of neutralization, higher glucose yields and no sugar degradation products (Balat, 2011; Sanchez and Cardona, 2008; Yang et al., 2011).

There are many factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks which can be divided in two groups: enzyme-related and substrate-related factors, though many of them are interrelated during the hydrolysis process (Alvira et al., 2010). Among these, are the combination of substrate composition and structure, pretreatment technology and severity, solid loading and enzyme dosage and efficiency are those with a great influence on the efficiency of cellulose hydrolysis (Alvira et al., 2010; Balat, 2011; Sun and Cheng, 2002; Yang et al., 2011).

In this work, the pretreatment temperature, enzyme dosage and solid loading were the variables studied, and the impact of these variables on enzymatic hydrolysis efficiency, by means of glucose concentration and yield, was evaluated applying response surface methodology. These three variables were selected due to their recognized main impact in enzymatic hydrolysis. The major goal of this work was thus to define the conditions providing concentrated glucose solutions from the materials tested (ER, WS and OP), once this feature is considered crucial to the global economy of sugar-based biorefineries. Moreover, the results of each individual feedstock were used to predict the conditions for enzymatic hydrolysis of pretreated mixtures (ER:WS:OP), towards maximal glucose recovery.

3.2 Materials and methods

3.2.1 Feedstock materials and pretreatment conditions

Eucalyptus residues (ER), consisting of bark, branches and leaves, wheat straw (WS) and olive tree pruning (OP), were collected in Mortágua, Elvas, and Ferreira do Alentejo (Portugal), respectively. These lignocelulosic materials were processed individually or in different mixtures (ER:WS:OP); 50:25:25; 25:50:25 and 25:25:50, represented by 50ER, 50WS and 50OP. The raw materials, containing less than 10% (w/w) moisture, were individually milled using a knife mill to particles size smaller than 6.0 mm and then stored to be used in the pretreatment step. The feedstock mixtures were prepared immediately before each pretreatment.

The autohydrolysis pretreatment was carried out in a 0.6-L stainless steel reactor (Parr Instruments Company, Moline, Illinois, USA) applying a liquid:solid ratio of 7:1 ($g_{water}/g_{feedstock}$). The pretreatment of individual feedstock (ER, WS and OP) was conducted under non-isothermal conditions at different temperatures (210, 220 or 230ºC) according to Table 3.1. After pretreatment the solid and liquid fraction were separated by hydraulic pressing. The solid fraction was washed at room temperature with two volumes of water and stored to be used in the enzymatic hydrolysis step.

The pretreatment methodology applied to individual feedstock was also used to process each feedstock mixture (50ER, 50WS and 50OP) only at the temperatures predicted by surface response analysis, using the results obtained for enzymatic hydrolysis of individual feedstock.

3.2.2 Enzymatic hydrolysis of pretreated solids

The enzymatic hydrolysis experiments were performed using the water insoluble solid (WIS) recovered after each hydrothermal pretreatment of individual feedstock (ER, WS and OP) and different enzyme dosage and solids loading according to the depicted in Table 3.1. Two commercial cellulolytic enzymes, Celluclast® 1.5L and Novozym® 188 (Novozymes, Denmark) were used as a cocktail (at a constant ratio of 9:1, respectively).The enzyme dosage used was 0.25, 0.50 and 0.75 (g_{enzyme}/g_{solids}), which corresponded to approx. 8-24 FPU/g_{solid} and 24-72 U β-glucosidase/g_{solid}). Three different solids loadings were assessed: 5, 12.5 and 20% (w/v). The enzymatic hydrolysis was performed in 0.05 M sodium phthalate buffer, pH 5.5, at 50°C, 140 rpm, for 96 h, in presence of sodium azide (0.08 % w/v) to avoid biological contamination. A control enzyme cocktail preparation was subjected to the same experimental conditions, but in the absence of solids. Samples were taken periodically and incubated at 100°C for 10 min. The supernatant was recovered by centrifugation at 13,000 rpm (10 min) (Beckman Coulter™ Microfuge® 18) and stored for the analysis of soluble sugars (incl. glucose and xylose).

Subsequently, the enzymatic hydrolysis of feedstock mixtures (50ER, 50OP and 50PO) were performed using the WIS obtained after their hydrothermal pretreatment. The same enzymatic hydrolysis methodology applied to individual feedstock was used to process each feedstock mixture. The highest solid loading, 20% (w/v), was applied, and the other two variables (pretreatment temperature and enzyme dosage) were predicted, by surface response analysis, towards maximal glucose recovery, using the results obtained with enzymatic hydrolysis of individual feedstock.

3.2.3 Analytical methods

The pretreated solids were characterized by quantitative acid hydrolysis, with 72% (w/w) H_2SO_4 for 60 min at 30°C, followed by hydrolysis with 4% (w/w) H_2SO_4 for 60 min at 121°C. The liquid fraction recovered after enzymatic hydrolysis were directly analysed in a high-performance liquid chromatography (HPLC) system (Agilent 1100 Series, Germany) equipped with a refractive index detector and an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The column temperature was set to 50ºC, and a mobile phase of 5 mM H_2SO_4 was used at 0.6 mL/min flow rate. Five-μL samples were injected and glucose and xylose were quantified after elution and chromatogram recording.

Enzymatic hydrolysis yields were determined according to Eq. 1:

$$\text{Glucose yield (\%)} = (C_{\text{Glucose 48h}} - C_{\text{Glucose 0h}})/(\text{Glucan } (g/g_{\text{solid}})*1.1*C_{\text{solids}}) * 100 \quad (1)$$

where:

$C_{\text{Glucose 48h}}$ is the glucose concentration (g/L) after 48 h of enzymatic hydrolysis

$C_{\text{Glucose 0h}}$ is the initial glucose concentration (g/L), carried over with the enzyme cocktail

1.1*Glucan (g/g_{solid}) is the glucose provided by the glucan content in the pretreated solids, by applying the hydration factor of glucan to glucose

C_{solids} is the solid loading used in the enzymatic hydrolysis (50, 125 or 200 g/L)

3.2.4 Surface response analysis

A 2^3 full factorial design, with 3 replicates in the center point and 6 centered face points consisting of 17 experiments (Table 3.1), was used in the optimization of enzymatic hydrolysis for each individual feedstock (ER, WS and OP) towards maximal glucose concentration and yield. Pretreatment temperature (X_1), enzyme dosage (X_2) and solid loading (X_3) were chosen for the independent variables (Table 3.1) Glucose concentration (Y_1) and glucose yield (Y_2) were used as the dependent output variables. A second order polynomial equation was used in order to develop an empirical model which correlated the responses to the independent variables, as shown in the Eq. 2:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+B_{23}X_2X_3+\beta_{11}X^2_1+\beta_{22}X^2_2+\beta_{33}X^2_3+\varepsilon \ (2)$$

where Y is the dependent output variables (glucose concentration or glucose yield); X the independent variable and the subscripts 1, 2 and 3 denote pretreatment temperature, enzyme dosage and solid loading, respectively; β_0 is the model intercept coefficient; β_1, β_2 and β_3 are the linear coefficients, $\beta_{12,}$ β_{13} and β_{23} are the second-order interaction coefficients between the variables, β_{11}, β_{22} and β_{33} are the quadratic coefficients the variables and ε is independent random error.

The Design Expert Statistical® 6.0 Software package (Stat Ease Inc., Minneapolis, USA) was employed to the statistical analysis of the second order model including analysis of variance (ANOVA). The experimental data were analysed by multiple regression analysis through the least square method. All the terms of the model were tested and verified statistically to confidence interval at 95% ($p \leq 0.05$), except for glucose yield of the WS, which was 90% ($p \leq 0.1$). Moreover, this Software was also used to provide the optimal condition for enzymatic hydrolysis of each individual feedstock and their mixtures. The response and contour plot was performed using Statistica for Windows^{TM} release 5.1 software (StatSoft Inc., USA).

3.2.5 Methodology applied to determine the optimal enzymatic hydrolysis conditions for different mixtures of the eucalyptus residues, wheat straw and olive tree

The results obtained for the enzymatic hydrolysis of each individual feedstock (ER, WS and OP) were used to predict the conditions of maximal glucose concentration and yield, in the range of variables studied, for the three feedstock mixtures (50ER, 50WS and 50OP) through two different approaches:

i) The Design Expert Statistical® 6.0 Software package predicted a single optimal operational condition of enzymatic hydrolysis for mixtures (equivalent to equal proportion).

ii) The Design Expert Statistical® 6.0 Software package predicted the optimal operational condition of enzymatic hydrolysis for each individual feedstock, which was applied for the mixtures, using the conditions of the most representative feedstock in each mixture.

3.3 Results and Discussion

3.3.1 Selection of experimental design parameters for the integration of pretreatment and enzymatic hydrolysis

The studied variables, namely pretreatment temperature (X_1); enzyme dosage (X_2) and solid loading (X_3) for enzymatic hydrolysis are considered to have major impact in glucose concentration and yield obtained from enzymatic hydrolysis of pretreated biomass (Alvira et al., 2010; Balat, 2011; Modenbach and Nokes, 2013; Sun and Cheng, 2002). The experimental range studied (lower and higher levels) was defined according to previous studies.

The pretreatment is a step required to alter the structure of lignocellulose, increasing glucan and xylan accessibility to the enzymatic hydrolysis. These structural modifications of the lignocellulose are highly dependent on the type of pretreatment employed (Sun and Cheng, 2002) and the severity of the pretreatment (Wyman et al., 2004), and these have a great effect on the enzymatic hydrolysis and subsequent steps. For the pretreament temperature, while 210°C was identified as the optimal temperature for maximal sugar recovery from the hemicellulose in the liquid fraction, for the individual feedstocks (ER, WS and OP), 230°C represented the temperature of extensive hemicellulose removal from the solid fraction and, maximal glucan content (Chapter 2 – Part A). Moreover, in the same work, the solids obtained from pretreatment of ER, WS and OP at 230°C revealed higher digestibility than at lower temperatures.

It is well known that the increase loading of cellulolytic enzymes in the process, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process (Sun and Cheng, 2002). In this work, the cellulolytic enzyme dosages varied from low enzyme levels

(0.25 g/g$_{solids}$, approx. 8 FPU/g$_{solids}$), aiming cost-competitive processes, up to high enzyme levels (0.75 g/g$_{solids}$, approx. 24 FPU/g$_{solids}$), for maximal enzymatic hydrolysis yields.

The solid loading is also known to have a great impact in process water economy of biochemical conversion of lignocellulose (Hahn-Hägerdal et al., 2006). The reduction of capital and production costs, by the use of smaller equipment and/or fewer reactors can be achieved with enzymatic hydrolysis performed at high-solids loadings (Modenbach and Nokes, 2013). Here, the solid loading for enzymatic hydrolysis was set between 5 and 20%, while 5% (w/v) solid loading is generally suitable to obtain higher hydrolysis yields, 20% (w/v) solid loading is considered the minimal to obtain glucose solutions with concentrations suitable for industrial bioconversion processes of lignocellulosic biomass, namely for bioethanol production (Larsen et al., 2008).

3.3.2 Glucose recovery after enzymatic hydrolysis of pretreated solids from individual feedstock

The independent variables pretreatment temperature (X_1), enzyme dosage (X_2) and solid loading (X_3) showed a major impact in the recovery of glucose (concentration and yield) for the studied range (Table 3.1). After 48 h of enzymatic hydrolysis of each feedstock, the glucose yield was higher than 40% under all the conditions tested, over 70% at the central points and over 95% at the most favourable conditions (230ºC, 75 g$_{enzyme}$/g$_{solids}$, 5% w/v solid loading) (Table 3.1). The enzymatic hydrolysis process was followed during 96 h, but the concentration and yields at 48 h were used for statistical analysis since,after this period, glucose increment was negligible.

The three feedstock (ER, WS an OP) assessed by enzymatic hydrolysis generated different glucose concentrations and yields under the same conditions. The different chemical composition of the lignocellulosic materials (Chapter 2 – Part A) was considered the most direct explanation for the results obtained. The highest values of glucose concentration were obtained for WS (Table 3.1) as a result from the highest glucan content obtained after pretreatment at 210 and 230ºC, comparing with ER and OP. The extensive solubilisation of the hemicellulose fraction contributed to this higher glucan content present in this pretreated feedstock (Chapter 2 – Part A). Moreover, agricultural residues have lower lignin content than woody materials and a more porous and readily accessible tissue structure, which is more susceptible to enzymatic hydrolysis (Carvalheiro et al., 2009; Martín-Sampedro et al., 2012). Also, other authors (Alvira et al., 2010) have reported that different types of biomass, including forestry and agricultural residues are slightly different in regard to composition, resulting in unique challenges in the enzymatic hydrolysis step of the conversion process.

Table 3.1 Glucose recovery (concentration and yield as the response factors studied) from eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) by using autohydrolysis and enzymatic hydrolysis as a function of the independent variables pretreatment temperature (X_1), enzyme dosage (X_2), solid loading (X_3) according to 2^3 full factorial (central composite face centred) experimental design.

	Assays	Temperature (°C) (X_1)	Enzyme (g/g_{solids}) (X_2)	Solid (%(w/v)) (X_3)	ER Glucose		WS Glucose		OP Glucose	
					(g/L)	(%)	(g/L)	(%)	(g/L)	(%)
Full factorial design 2^3	1	210(-1)[a]	0.25(-1)	5.0(-1)	13.71	49.82	22.40	78.82	14.60	64.84
	2	230(+1)	0.25(-1)	5.0(-1)	17.06	58.60	30.55	94.86	21.48	91.93
	3	210(-1)	0.75(+1)	5.0(-1)	16.87	61.33	28.34	99.73	18.44	81.90
	4	230(+1)	0.75(+1)	5.0(-1)	28.27	97.10	31.28	97.13	23.99	102.65
	5	210(-1)	0.25(-1)	20.0(+1)	44.87	40.77	68.38	60.15	53.74	59.68
	6	230(+1)	0.25(-1)	20.0(+1)	61.36	52.69	87.82	68.18	75.83	81.13
	7	210(-1)	0.75(+1)	20.0(+1)	60.44	54.92	87.62	77.07	72.18	80.15
	8	230(+1)	0.75(+1)	20.0(+1)	75.46	64.80	103.86	80.63	83.64	89.48
Central points	9	220(0)	0.50(0)	12.5(0)	54.01	78.11	72.53	92.60	50.28	87.30
	10	220(0)	0.50(0)	12.5(0)	53.68	77.63	75.26	96.08	50.56	87.78
	11	220(0)	0.50(0)	12.5(0)	50.52	73.06	73.56	93.90	51.56	89.52
Model Centered face	12	210(-1)	0.50(0)	12.5(0)	29.56	42.99	59.84	84.21	40.98	72.81
	13	230(+1)	0.50(0)	12.5(0)	43.97	60.41	71.67	89.02	52.15	89.27
	14	220(0)	0.25(-1)	12.5(0)	43.42	62.79	55.40	70.72	49.06	85.18
	15	220(0)	0.75(+1)	12.5(0)	53.36	77.16	55.90	71.37	49.09	85.22
	16	220(0)	0.50(0)	5.0(-1)	22.12	79.95	32.62	104.10	22.95	99.60
	17	220(0)	0.50(0)	20.0(+1)	88.57	80.05	108.69	86.73	79.34	86.09

[a] Real variables (coded variables)

Among the three feedstocks, the glucose concentration obtained after enzymatic hydrolysis was higher for WS at higher severity of pretreatment, which was apparently related with the lower hemicellulose content of that solid (Chapter 2 – Part A), and more extensive re-localization of lignin and alteration of cellulose structure under those conditions (Cara et al., 2008; Kabel et al., 2007). Thus, the pretreatment temperature had a pronounced effect on the digestibility of cellulose present in the lignocellulosic materials assessed. Under the most favourable conditions (230ºC, 75 (g_{enzyme}/g_{solids}), 5% (w/v) solid loading), the temperature employed in pretreatment promoted virtually complete enzymatic hydrolysis of cellulose (>95% glucose yield). The pretreatment used, autohydrolysis, and the temperature range studied has thus demonstrated that this technology is suitable for integration in biorefining processes with subsequent efficient enzymatic hydrolysis (Chapter 2 - Part A).

The solid loading was another parameter studied because it usually significantly affects enzymatic hydrolysis yields and also because high-solids loadings are required to render the lignocellulosic conversion process economically feasible (Koppram et al., 2014; Modenbach and Nokes, 2013). An enzymatic hydrolysis process considered at “high solids” is performed at high solids/liquid ratio, in such that very little to no free water is present in the slurry, i.e. the solids loading is higher than 15% (w/w) (Modenbach and Nokes, 2013). Enzymatic hydrolysis performed at high-solids loadings offers several advantages over low- and moderate-solids loadings, the main one consists of higher final sugar concentrations achieved. In theory, higher sugar concentrations provide cost-effective downstream processing due to significant water and energy economy, as in the case of second-generation bioethanol production where higher ethanol concentrations (>4% v/v) significantly reduces energy required, and thus costs, associated with the distillation process (Hahn-Hägerdal et al., 2006). On contrary, high solid loading has major disadvantages in terms of rheology constrains and the inhibition of enzymatic reactions by their product (Knutsen and Liberatore, 2009). As expected, the increase in solid loading for enzymatic hydrolysis increased final glucose concentration but reduced the conversion yield. This effect was more pronounced with ER than with WS and OP, decreasing from more than 95% yield at 5% (w/v) solid loading (and 230ºC and 75 g_{enzyme}/g_{solids}) to approx. 65, 80 and 90% yield, respectively. The higher amount of soluble sugars present in WS and OP and the higher recalcitrance of ER may explain the results obtained.

Finally, enzyme dosage was the third parameter studied in enzymatic hydrolysis of pretreated solids of ER, WS and OP, as increasing the dosage of cellulolytic enzymes in the process, to a certain extent, can enhance the hydrolysis rate and yield, but would significantly increase the cost of the lignocellulosic biorefinery processes (Sun and Cheng, 2002). A cellulase dosage of 10 FPU/$g_{cellulose}$ is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) (Sun and Cheng, 2002) and at an enzyme cost compatible with scaling-up towards commercialization. Most of the studies report enzyme loadings varying from 7 to 33 FPU/$g_{substrate}$, depending on type and concentration of substrates (Sun and Cheng, 2002). In this study, the 0.25, 0.50 and 0.75 (g_{enzyme}/g_{solids}) corresponded to approx. 8, 16 and 24 FPU/g_{solids} respectively, equivalent to 10-60FPU/$g_{cellulose}$. As expected, the higher enzyme loading (0.75 g_{enzyme}/g_{solids}) provided hydrolysis yields over 95%, under the most favourable conditions assessed (230ºC and 5% w/v solid loading). However, the lower enzyme loading (0.25 g_{enzyme}/g_{solids}) applied to 5% (w/v) solids pretreated at 230ºC dramatically decreased glucose yield in the case of ER (<60%), but did not generate a great impact in enzymatic hydrolysis of pretreated WS and OP, which maintained glucose yields over 90%.

In general, glucose concentration and yield were higher at higher pretreatment temperature and enzyme dosage; the higher solid loading promoted higher glucose concentration but lower glucose yield. Others authors have also observed that the substrate concentration affected the yield and initial rate of cellulose enzymatic hydrolysis (Sun and Cheng, 2002). Indeed, high substrate concentration might promote substrate and product inhibition, with the subsequent significant decrease of the hydrolysis rate (Yang et al., 2011). Although the glucose concentration is an important response factor, the conversion yield is a relevant factor with impact on the economy of the process. Low enzymatic hydrolysis yields and rates have a great impact on the overall cellulose-to-ethanol conversion efficiency, and thus have been identified as bottlenecks of bioethanol production processes during the past decade (Yang et al., 2011).

This study, targeting the optimization of enzymatic hydrolysis of ER, WS and OP and their mixtures, was integrated with previous optimization of pretreatment (autohydrolysis) for the same materials (Chapter 2 – Part A) and their mixtures (Chapter 2 – Part B). Therefore, it is not surprising that the results obtained for enzymatic hydrolysis are, in general, superior to those obtained by other authors with similar raw materials. Romaní et al., (2010) have studied the pretreatment of *Eucalyptus globulus* wood in aqueous media (autohydrolysis) under non-isothermal conditions to reach maximal temperatures in the range of 195–250ºC. The objective was to assess the effect of pretreatment severity on the fractionation of wood and on the susceptibility of processed solids towards enzymatic hydrolysis. The experiments were carried out under the same operational conditions for all the pretreated solids: liquid-solid ratio was 20 g/g (5% w/v solid loading) and enzyme to substrate ratio was 10.3 FPU/g_{solids}, and 10 U β-glucosidase/FPU, which is similar to the lowest levels of substrate loading and enzyme dosage applied in the present study. These authors observed that harsher autohydrolysis conditions resulted in higher glucose concentrations in the enzymatic hydrolysis of pretreated solids (up to 35 g/L), allowing virtually complete cellulose conversion into glucose for materials pretreated above 210ºC. However, these authors did not perform assays at higher solid loading.

Using a statistical approach, Pérez et al., (2008) have studied the optimization of conditions for liquid hot water pretreatment of WS to enhance sugar recovery by enzymatic hydrolysis. The enzymatic hydrolysis assays were performed at 50ºC for 72 h, in 0.05 M sodium citrate buffer (pH 4.8), using a low solid loading of 4% (w/v). The enzyme loading was 15 FPU/g_{solids} of Celluclast® 1.5L and 15 U β-glucosidase/g_{solids} of Novozym® 188, which is in the range of the dosage applied in this work. These authors observed a 79.8%-yield for enzymatic hydrolysis of the solid pretreated at 188ºC (for 40 min) and 90.6%-yield for pretreatment at 214ºC (for 2.7 min), demonstrating that the pretreatment severity factor is relevant for enzymatic hydrolysis.

Cara et al., (2007) have studied the influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass. The enzymatic hydrolysis of pretreated solids was performed using the same commercial enzymes used in this work (Celuclast® 1.5L at 15 FPU/g_{solids} and Novozyme® 188 at 15 U β-glucosidase/g_{solid}) at solid loading in the range of 2-30% (w/v). These authors have studied the influence of the substrate concentration on the enzymatic hydrolysis yield and on glucose concentration, and verified that the concentration of this monosaccharide also increased as a function of substrate concentration. On the other hand, the hydrolysis yield after 72 h decreased as a function of substrate concentration, reaching 76.2% and 49.9% for 2 and 30% of solid loading, respectively.

The present work covered the most relevant factors controlling the efficiency of enzymatic hydrolysis for ER, WS and OP, towards the maximization of glucose concentration and yield. At the highest solid loading assessed (20% w/v), it was possible to obtain 89, 109, and 84 g/L of glucose by enzymatic hydrolysis of WIS from pretreated ER, WS and OP, respectively (assays 8 and 17, Table 3.1), corresponding to 80-89%-yield.

3.3.3 Response surface methodology for maximal recovery of glucose from individual feedstock

From a general analysis it was possible to select the factors and second-order interactions that were significant at the 95% confidence level. The effects and standard errors for all the dependent variables from the experimental matrix were estimated.

An ANOVA of the significant values from Student's t-distribution was made (data not shown) to determine if the regression equation was statistically significant. To improve the models the insignificant models terms were removed from the models. The ANOVA of the quadratic models for glucose concentration and yield are shown in Tables 3.2 and 3.3, respectively. The models were adjusted using only the significant independent variables.

Tables 3.2 and 3.3 show the ANOVA of the quadratic model adjustment to data, where the total error was dismembered into lack-of-fit and pure error. This procedure is valid since the replications were performed at the center point in order to obtain an estimate of the random error (pure error). The values estimated indicate that the models were significant in the region studied. Although the model for glucose concentration from WS and OP presented a slight lack-of-fit, no impairment of the model was observed due to the fact that for all models the p-values were <0.0001, which shows that the models are strongly significant at 99% of confidence level.

By the table 3.2 and 3.3 also can be see the significant effects on the two responses variable (glucose concentration and yield) from the enzymatic hydrolysis of WIS from ER, WS and OP. For the three individual feedstocks studied, in the case of glucose concentration, the first-order effects of pretreatment temperature, enzyme dosage and solid loading were significant at a 95% confidence level. This clearly demonstrates the relevance of these factors in the enzymatic hydrolysis performance aiming the maximization of glucose concentration. For WS, the pretreatment temperature was statistically significant only at 90% confidence level. The interaction between the variables was just observed for glucose concentration from OP. Table 3.2 shows that the interaction between solid loading with pretreatment temperature and enzyme dosage was, in this case, significant at a 95% confidence level. So, it is evident that there was a quadratic relationship between the independent variables (factors and their interactions) and the response variable (glucose concentration and yield), indicating that all the factors were statistically significant, with a great confidence interval.

Table 3.2 Analysis of variance (ANOVA) for response surface reduced quadratic model of glucose concentration from eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) by using autohydrolysis and enzymatic hydrolysis as a function of pretreatment temperature (X_1), enzyme dosage (X_2) and solid loading (X_3).

	Source		Sum of Squares	DF	Mean Square	F-Value	*p*-value
ER	**Model**		6778.28	4	1694.57	50.33	<0.0001
		X_1	368.16	1	368.16	10.93	0.0063
		X_2	291.44	1	291.44	8.66	0.0123
		X_3	5413.94	1	5413.94	160.79	<0.0001
		X_1^2	704.74	1	704.74	20.93	0.0006
	Residual		404.04	12	33.67		
	Lack of Fit		396.61	10	39.66	10.68	0.0886
	Pure Error		7.43	2	3.71		
	Cor. Total		7182.32	16			
WS	**Model**		10951.29	4	2737.82	75.76	<0.0001
		X_1	343,33	1	343.33	9.50	0.0095
		X_2	180.31	1	180.31	4.99	0.0453
		X_3	9683.91	1	9683.91	267.96	<0.0001
		X_2^2	743.73	1	743.73	20.58	0.0007
	Residual		433,67	12	36.14		
	Lack of Fit		429.87	10	42.99	22.61	0.0431
	Pure Error		3.80	2	1.90		
	Cor. Total		11384.96	16			
OP	**Model**		7561.11	6	1260.19	175.64	<0.0001
		X_1	326.66	1	326.66	45.53	<0.0001
		X_2	106.38	1	106.38	14.83	0.0032
		X_3	6931.75	1	6931.75	966.14	<0.0001
		X_1^2	91.08	1	91.08	12.70	0.0052
		$X_1 X_3$	55.72	1	55.72	7.77	0.0192
		$X_2 X_3$	49.51	1	49.51	6.90	0.0253
	Residual		71.75	10	7.17		
	Lack of Fit		70.84	8	8.85	19.52	0.0496
	Pure Error		0,91	2	0.45		
	Cor. Total		7632.86	16			

Significant at 95% confidence level for all feedstock mixture.

Table 3.3 Analysis of variance (ANOVA) for response surface reduced quadratic model of glucose yield from eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) by using autohydrolysis and enzymatic hydrolysis as a function of pretreatment temperature (X_1), enzyme dosage (X_2) and solid loading (X_3).

	Source		Sum of Squares	DF	Mean Square	F-Value	p-value
ER	**Model**		3190.01	5	638.00	15.04	0.0001
		X_1	701.54	1	701.54	16.54	0.0019
		X_2	821.58	1	821.58	19.37	0.0011
		X_3	286.87	1	286.87	6.76	0.0247
		X_1^2	1331.11	1	1331.11	31.38	0.0002
		X_3^2	162.97	1	162.97	3.84	0.0758
	Residual		466.63	11	42.42		
	Lack of Fit		451.10	9	50.12	6.45	0.1413
	Pure Error		15.54	2	7.77		
	Cor. Total		3656.64	16			
WS	**Model**		2240.49	5	448.10	17.70	<0.0001
		X_1	89.01	1	89.01	3.55	0.0862
		X_2	283.11	1	283.11	11.30	0.0064
		X_3	1038.01	1	1038.01	41.42	<0.0001
		X_2^2	828.47	1	828.47	33.06	0.0001
		X_3^2	185.62	1	185.62	7.41	0.0199
	Residual		273.67	11	25.06		
	Lack of Fit		358.48	9	29.94	9.66	0.0973
	Pure Error		6.20	2	3.10		
	Cor. Total		2516.16	16			
OP	**Model**		1640.41	4	410.10	20.24	<0.0001
		X_1	903.82	1	903.82	44.61	<0.0001
		X_2	320.88	1	320.88	15.84	0.0018
		X_3	197.14	1	197.14	9.73	0.0089
		X_1^2	218.57	1	218.57	10.79	0.0065
	Residual		243.11	12	20.26		
	Lack of Fit		240.37	10	24.04	17.58	0.0550
	Pure Error		2.73	2	1.37		
	Cor. Total		1883.51	16			

ER and OP significant at a 95% confidence level, WS significant at a 90% confidence level.

Table 3.4 shows the statistical models for glucose concentration and yield of ER (Y_2 and Y_3), WS (Y_4 and Y_5) and OP (Y_6 and Y_7), and their respective coefficients of determination (R^2). The independent variables X_1, X_2 and X_3 correspond to pretreatment temperature, enzyme dosage and solid loading, respectively. The coefficient of determination (R^2) indicates the percentage of the total variation around the average could be explained by the regression (Table 3.4).

Table 3.4 Polynomial models for glucose concentration from ER (Y_1); glucose yield from ER (Y_2); glucose concentration from WS (Y_3); glucose yield from WS (Y_4); glucose concentration from OP (Y_5); glucose yield from OP (Y_6) ant the respective coefficient of determination (R^2).

Polynomial models	R^2
$Y_1 = 52.24008 + 6.06766 * X_1 + 5.39849 * X_2 + 23.26787 * X_3 - 13.08245 * X_1^2$ (2)	0.94
$Y_2 = 73.43965 + 8.37579 * X_1 + 9.06412 * X_2 - 5.35601 * X_3 - 20.96467 * X_1^2 + 7.33554 * X_3^2$ (3)	0.96
$Y_3 = 70.59581 + 5.85947 * X_1 + 4.24629 * X_2 + 31.11899 * X_3 - 13.43949 * X_2^2$ (4)	0.99
$Y_4 = 90.14087 + 2.98348 * X_1 + 5.32079 * X_2 - 10.18830 * X_3 - 16.53940 * X_2^2 + 7.82882 * X_3^2$ (5)	0.89
$Y_5 = 50.40664 + 5.71545 * X_1 + 3.26163 * X_2 + 26.32822 * X_3 - 4.70321 * X_1^2 + 2.63913 * X_1 * X_3 + 2.48777 * X_2 * X_3$ (6)	0.86
$Y_6 = 88.66971 + 9.50696 * X_1 + 5.66462 * X_2 - 4.44000 * X_3 - 7.28564 * X_1^2$ (7)	0.87

The first-order effects of the pretreatment temperature, enzyme dosage and solid loading had a positive effect in the enzymatic hydrolysis, favoring the glucose concentration from ER, WS and OP (Y_2, Y_4 and Y_6). For the glucose yield (Y_3, Y_5 and Y_7), pretreatment temperature and enzyme dosage also had a positive effect in this dependent variable, however for ER and OP the effect was observed at a 95% confidence level and for WS at 90%. However, the solid loading presented a negative effect on glucose yield, i.e. the lower solid loading favors the enzymatic hydrolysis yield, which is in agreement with the observation of others authors (Cara et al., 2007). The interaction observed between temperature pretreatment and solid loading (X_1*X_3) and enzyme dosage and solid loading (X_2*X_3) for glucose concentration from OP implies that these variables act synergistically to increase glucose concentration, once that in both cases the interaction presented positive values.

The equations (2-7) were used in STATISTICA®5.1 Software package (Statsoft, USA) in order to get the response surface model for glucose concentration and yield, and visualize the trend of maximizing these responses. Thus, the response surfaces were drawn as three-dimensional plots of two factors (temperature pretreatment and enzyme dosage) while the other factor (solid loading) was kept constant at the highest level (+1) (Fig. 3.1a-f).

The response surfaces plotted for enzymatic hydrolysis of pretreated ER (Fig. 3.1a and 3.1b) at the high level of solid loading show that the maximal glucose recovery can be achieved using the highest level of enzyme dosage and a pretreatment temperature close to the central point. On contrary, for WS (Fig. 3.1c and 3.1d), the conditions promoting maximal glucose concentration and yield correspond to the highest pretreatment temperature and to an enzyme dosage close to the central point. For OP, the highest levels of enzyme dosage and pretreatment temperature are required to obtain a maximal glucose concentration and yield, as suggested by the surface responses (Fig. 3.1e and 3.1f).

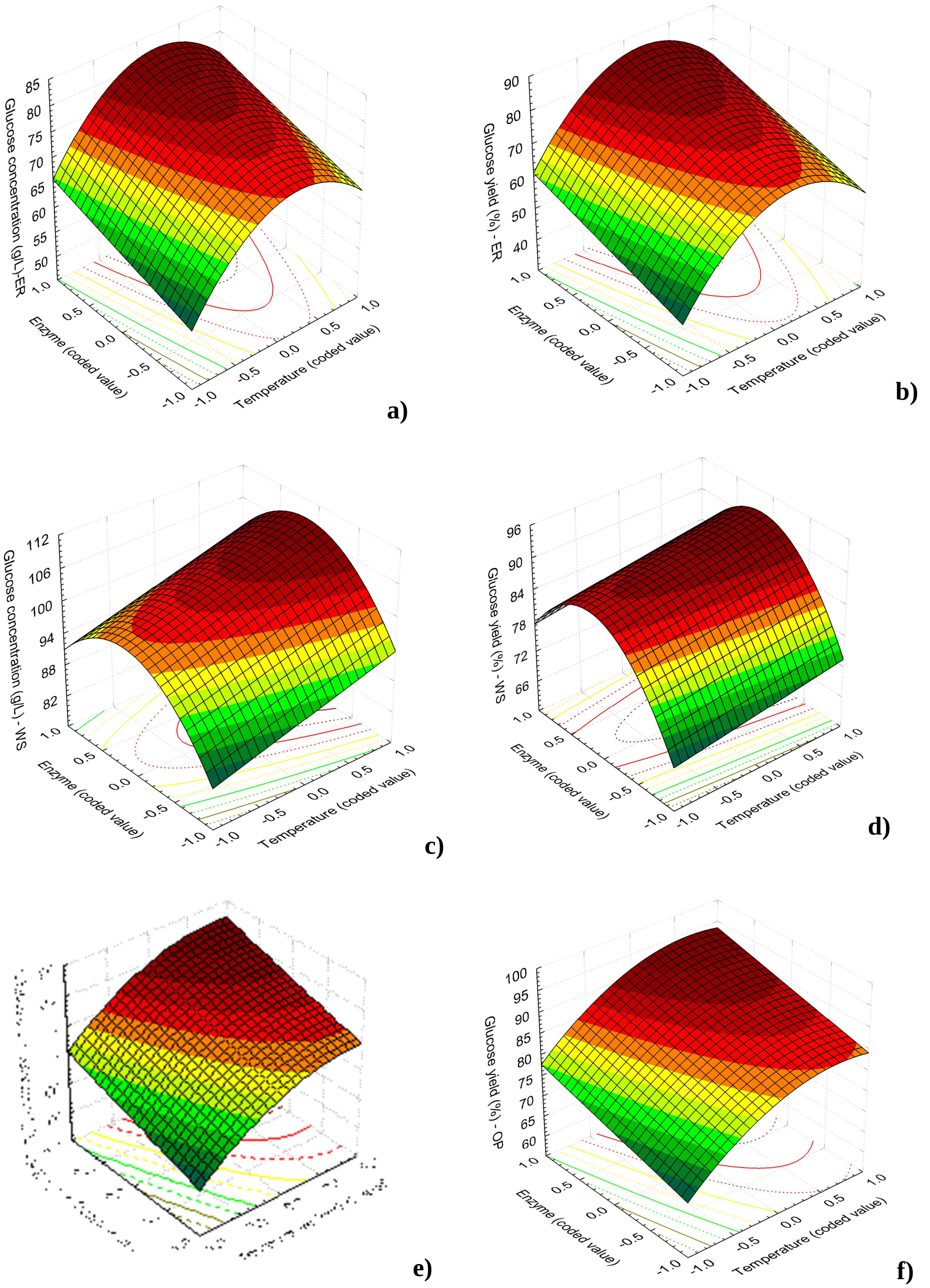

Figure 3.1 Response surface and contour plots of glucose concentration (a, c and e) and glucose yield (b, d and f) after enzymatic hydrolysis of pretreated eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) as a function of pretreatment temperature (X_1), enzyme dosage (X_2) and solid loading (X_3) (solid concentration was kept constant at coded level of +1).

After the analysis of results obtained by response surface methodology for the optimization of enzymatic hydrolysis of ER, WS and OP, assessed by the responses glucose concentration and yield, the optimal conditions to maximize glucose concentrations using the highest solid loadings (20% w/v) were determined with support of Design Expert Statistical® 6.0 Software package (Stat Ease Inc., Minneapolis, USA). Different values were observed for the optimal condition for ER, WS and OP individually pretreated, in the range studied. The enzymatic hydrolysis of ER can generate the maximal glucose concentration of 81.6 g/L, with 222°C of pretreatment temperature and 0.75 (g_{enzyme}/g_{solids}) of enzyme dosage. In the case of enzymatic hydrolysis of WS, the maximal glucose concentration, 107.9 g/L, can be achieved using 230°C of pretreatment temperature and 0.54 (g_{enzyme}/g_{solids}) of enzyme dosage. Finally, the enzymatic hydrolysis of OP, can achieve a maximal glucose concentration of 86.1 g/L, at 228ºC pretreatment temperature and 0.75 (g_{enzyme}/g_{solids}) enzyme dosage.

3.3.4 Prediction of glucose concentration for mixtures of the eucalyptus residues, wheat straw and olive tree pruning using the statistical models of individual feedstock

The optimal conditions for enzymatic hydrolysis of the mixtures 50ER, 50WS and 50OP at 20% (v/w) of solid loading were determined with the results and respective models obtained for individual feedstock (ER, WS and OP) (Table 3.5). The predicted glucose concentrations and the respective experimental results obtained revealed that 85 g/L can be achieved, independently of the feedstock proportions in the mixtures and the different optimized conditions.

Two different conditions were studied for each of the three mixtures of ER, WS and OP, as a function of the two different approaches chosen. In the first approach, the Design Expert Statistical® 6.0 Software package predict a single optimal operational condition of enzymatic hydrolysis (assays A, C and E). In the second approach, the optimal conditions obtained for each individual feedstock were tested for the different feedstock mixtures (assays B, D and F).

Table 3.5 Predicted and experimental values of the glucose concentration at optimum conditions of pretreatment temperature and enzyme dosage for different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) at high solid loading (20% w/v).

Assays	Feedstock mixture	Temperature (ºC)	Enzyme (g/g_{solids})	Predicted Glucose (g/L)	Experimental Glucose (g/L)
A	50ER	224	0.63	85.55	86.20±1.47
B		222	0.75	85.25	88.86±1.46
C	50WS	224	0.63	91.51	92.18±1.98
D		230	0.54	91.62	89.61±2.08
E	50OP	224	0.63	86.43	85.25±0.72
F		228	0.75	86.80	85.34±1.61

The lower difference between the glucose concentration predict by the model and that obtained experimentally for the feedstock mixtures was inferior to 5% independent of the approach chosen. These results demonstrated the consistence of the models used. Thus, the models of individual feedstock can be used to predict results for different combinations of feedstock with sufficient confidence. The use of models for enzymatic hydrolysis of ER, WS and OP to predict enzymatic hydrolysis of their mixtures, can bring benefits to biomass processing with special relevance in the Southern European countries, due to the possibility of working with several different mixtures in a biorefinery framework.

The low difference between the results obtained with the two approaches, i.e. the conditions of the feedstock predominant in each mixture or a single condition for all feedstock mixtures (comparing the assays A and B, C and D and E and F) show the flexibility of the enzymatic hydrolysis conditions for processing feedstock mixtures. Thus, the use of conditions of the most representative feedstock (assays A, C and B) or the use of a common condition of all mixtures of ER, WS and OP (B, D and E) is a function of cost analysis and a trade-off between the enzyme dosage and the energy consumption for pretreatment, but also the purpose of the liquid fraction obtained after autohydrolysis, since high temperature pretreatment generate larger amounts of degradation products (furfural, 5-(hydroxymethyl)furfural and phenolic compounds) (Carvalheiro et al., 2009).

The results obtained with the feedstock mixtures studied, namely 50ER, 50WS and 50OP bring potential benefits to biorefineries operating in Southern European countries, mainly with respect to biomass supply, minimizing problems associated to feedstock availability, seasonality, storage and preservation. Moreover, the use of mixtures around the year may not require the change of operational conditions, which increases flexibility.

3.4 Conclusions

The optimization of enzymatic hydrolysis of hydrothermally pretreated ER, WS and OP through statistical analysis was crucial to achieve more than 85% glucose yield either for individual feedstock or for several mixtures containing these lignocellulosic materials at 20% (w/v) solid loading. Together with the optimization of pretreatment (autohydrolysis) conditions (Chapter 2), this work on the optimization of cellulose enzymatic hydrolysis demonstrated the feasibility of processing different mixtures of lignocellulosic materials, including agricultural residues and hardwoods, towards a sugar platform for subsequent biological or chemical conversion. The results obtained potentiate, at different levels, the operation of a multi-feedstock biorefiniery: demonstrate glucose recovery yields higher than 80% from the cellulosic fraction of ER, WS and OP and their mixtures after hydrothermal pretreatment and enzymatic hydrolysis; demonstrate the ability of processing different mixtures at similar yields (substrate flexibility), which may mitigate seasonal variability of biomass availability and supply; feedstock-dependent or -independent operational conditions may be applied, with constant operational conditions still promoting more than 80% of enzymatic hydrolysis yields, thus demonstrating process flexibility; the cellulosic hydrolysates at 80-110 g/L glucose concentration may be directly processed in biological or chemical conversion processes for the productions of biofuels, biochemicals and biomaterials.

3.5 References

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CHAPTER

4

BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC MIXTURES

Bioethanol production from mixtures of eucalyptus residues, wheat straw and olive tree pruning by SHCF and SSCF at high solid loading using the recombinant xylose-fermenting *Saccharomyces cerevisiae* BH42

Abstract

The economic viability of ethanol production from lignocellulosic materials requires inexpensive and stable feedstock supply, operations at high-solid loading to obtain final ethanol concentrations over 4% (w/v), which is compatible with cost-competitive ethanol recovery processes, and the conversion of both the cellulosic and hemicellulosic fractions to maximize ethanol yield. Therefore, the use of robust xylose-fermenting *Saccahomyces cerevisiae* strains able to efficiently convert hexoses and pentoses into ethanol is mandatory. Moreover, the operation of multi-feedstock biorefineries can mitigate limited biomass availability in certain regions. In the present work, mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) were processed by hydrothermal pretreatment (autohydrolysis), and subject to bioconversion at high solid loading (20% w/v) by separate hydrolysis and (co)-fermentation (SH(C)F) and simultaneous saccharification and (co)-fermentation (SS(C)F) using commercial enzymes and recombinant xylose-fermenting *Saccharomyces cerevisiae*. The influence of using detoxified hemicellulosic hydrolysate obtained from autohydrolysis with or without chemical post-hydrolysis was valued. Using different feedstock mixtures and process configurations, the final ethanol concentrations and yields always reached around 40 g/L and 0.3 g/g total sugars, respectively, after 48 h fermentation, thus suggesting that the utilization of these mixtures in industrial ethanol production might be feasible through the approached proposed.

Keywords

Autothydrolysis, bioethanol, biorefinery; hemicellulosic hydrolysate; recombinant *Saccharomyces cerevisiae*; xylose fermentation

Highlights

- Hydrothermal pretreatment, enzymatic hydrolysis at 20% (w/v) solid loading and fermentation, using a recombinant xylose-fermenting *Saccharomyces cerevisiae*, generated more than 40 g/L ethanol under both SH(C)F and SS(C)F of feedstock mixtures.
- SHCF promoted higher ethanol productivities in the fermentation steps.
- SSCF promoted higher xylose co-fermentation with recombinant *Saccharomyces cerevisiae.*
- The co-fermentation process was inhibited by hemicellulosic hydrolysates.
- The xylose co-fermentation promoted an increase in the ethanol concentration.

Abbreviations

GlcOS, glucooligosaccharides; XOS, xylooligosaccharides; AOS, arabinooligosaccharides; HMF, 5-(hydroxymethyl)furfural, AHH, autohydrolysis hemicellulose hydrolysate; PHH, post-hydrolysis hemicellulose hydrolysate.

4.1 Introduction

Second generation (2G) bioethanol, or lignocellulosic ethanol, is reaching commercialization. The major driving forces for the development of the lignocellulosic ethanol technology have been associated to the increasing use of transportation fuels worldwide, and include the dependence on oil and the increasing prices of fossil fuels and the increasing emission of greenhouse gases and its impact on climate change. The regulatory framework put in practice by policy makers, namely with established targets of biofuels blending in conventional fuels (e.g. EU Directive 2009/CE/28) is seen as an opportunity to commercialize the technology in certain regions.

In Southern European countries the availability of a single feedstock is limited and the use of feedstock mixtures, including those combining agricultural residue and hardwood residues, can bring additional benefits in the operation of a 2G bioethanol biorefinery in this region. Feedstock mixtures comprising eucalyptus residues (ER), including bark, branches and leaves, wheat straw (WS) and olive tree pruning (OP) are attractive feedstocks to be use in a Southern-European biorefineries due to their availability and high saccharide content (Chapter 2 – Part A). The use of these feedstocks in mixtures can minimize problems related with the individual feedstock availability, seasonality and storage and can promote the continuous operation of a biorefinery in this region, preferentially without changing processing conditions. In fact, mixtures of lignocellulosic materials containing those feedstocks were successfully converted into monosaccharides under similar conditions of hydrolthermal pretreatment (Chapter 2 – Part B) and enzymatic hydrolysis (Chapter 3).

The effective conversion of all the sugars available in lignocellulosic biomass and the high final ethanol concentration and yield obtained in the fermentation process have been considered key-factors for economic viability of 2G bioethanol (Wingren et al., 2003). The lignocellulosic biomass is composed of 60-80% polysaccharides, which can be hydrolyzed to obtaining varieties of sugars. Cellulose is recovered in the solid fraction after hydrothermal pretreatment and is the major source of sugars. Its enzymatic hydrolysis generates mainly glucose. Under hydrothermal pretreatment hemicellulose is partially hydrolysed and mainly recovered in the liquid fraction, and the resulting hemicellulosic hydrolysate is usually composed of xylose, arabinose, mannose, galactose and glucose (Gírio et al., 2010; Sun and Cheng, 2002).

The bioconversion steps, enzymatic hydrolysis (and fermentation), should be initiated at high solid loading, preferentially combining the cellulosic (solid) fraction with the hemicellulosic fraction (Gírio et al., 2010). The bioconversion of the whole slurry obtained after hydrothermal preatreatment can also contribute to the economy of the process since it avoids separations processes.

The integrated upgrading of the cellulosic and hemicellulosic sugars have been carried out by two distinct processes, namely separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF) (Gírio et al., 2010; Olsson et al., 2006). The combination of enzymatic hydrolysis (saccharification) and (co-)fermentation in a single step makes the SS(C)F process very attractive from an economic point of view, not only because two operations are performed in a single bioreactor, but also because it reduces the effect of product inhibition of enzymatic hydrolysis by continuous sugar consumption in the fermentation (Olofsson et al., 2008). Moreover, the low concentration of glucose in the medium, provided continuously at low rates by hydrolysis step, provides to the SSCF process an additional advantage of favouring the co-transport of xylose into the cell (Fonseca et al., 2011; Olofsson et al., 2008). However, the SSCF process is carried out in sub-optimal temperature conditions for each stage (Taherzadeh and Karimi, 2007). Furthermore, the SHCF, which as the name implies is performed in two separate operations, enables the use of the optimum temperature in each step, 50ºC for enzymatic hydrolysis and 35ºC for fermentation (Harun et al., 2001). However, it has the drawback of being inhibited by the enzymatic hydrolysis reaction product which accumulates during the process, which decreases the productivity and yield of hydrolysis. However, this effect is not significant when using the most recent commercial cellulolytic cocktails.

Lignocellulosic ethanol processes performed at high-solid loading (>15% solids, w/w) contribute to both energy and water savings, namely by less energy-demanding processes for ethanol recovery at high ethanol concentrations, by lower water consumption and by lower costs associated with wastewater treatment (Modenbach and Nokes, 2013).

Although extensive research has been carried out to develop the 2G bioethanol technology, the efficient fermentation of pentoses by *S. cerevisiae*, especially under co-fermentation with glucose is still a challenge to overcome (Fonseca et al., 2011). In fact, *S. cerevisiae*, the yeast commonly used in industrial ethanol processes, efficiently ferment hexoses but is unable to metabolize pentoses (Hahn-Hägerdal et al., 2007). Xylose is the predominant sugar of most hemicelluloses and is present in the liquid fraction on many hemicellulosic hydrolysates from hydrothermal and acidic pretreatments (Gírio et al., 2010). Accordingly, an approach that has been widely used is the heterologous expression of metabolic pathways in *S. cerevisiae* conferring the pentose-fermentation capacity, with focus on xylose (Karhumaa et al., 2007a, 2007b; Kuyper et al., 2005). The genes *XYL1* and *XYL2* coding xylose reductase (XR) and xylitol dehydrogenase (XDH), respectively, or *XylA* coding xylose isomerase (XI), in combination with the endogenous gene *XKS1* encoding xylulokinase (XK) have been introduced in laboratory and industrial *S. cerevisiae* strains, allowing for xylose utilization for growth and ethanol production (Eliasson et al., 2000; Garcia-Sanchez et al., 2010; Kuyper et al., 2005; Matsushika et al., 2009a, 2009b; Sodereger et al., 2004; Wahlbom et al., 2003; Wisselink et al., 2009).

The present work envisages to demonstrate 2G bioethanol production from mixtures of lignocellulosic materials, ER, WS and OP (ER:WS:OP; 50:25:25; 25:50:25; 25:25:50), combining hydrothermal pretreatment (autohydrolysis), enzymatic hydrolysis at high solid loading (20% w/v) and co-fermentation of glucose and xylose, through SHCF and SSCF, employing commercial enzymes and the recombinant xylose-fermenting *S. cerevisiae* BH42. Co-fermentation process using chemically defined liquid medium containing xylose, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were performed for comparative purposes.

4.2 Materials and Methods

4.2.1 Feedstocks

Three different feedstock combinations of eucalyptus residues (ER) consisting of bark, branches and leaves (Mortágua, Portugal), wheat straw (WS) (Elvas, Portugal) and olive tree pruning (OP) (Ferreira do Alentejo, Portugal) were used (ER:WS:OP; 50:25:25; 25:50:25 and 25:25:50, represented as 50ER, 50WS and 50OP, respectively). Each feedstock was individually ground with a knife mill to particles smaller than 6.0 mm and three different combinations of feedstock were prepared and characterized. On average the feedstock mixtures presented the followed composition in dry weight basis (%): 50ER - glucan, 32.7; xylan, 17.9; arabinan; 2.2, acetyl groups, 4.2; Klason lignin 23.5, ash, 3.9 and others, 10.6, including 4.5 of soluble sugars; 50WS - glucan, 31.7; xylan, 19.2; arabinan; 2.7, acetyl groups, 3.6; Klason lignin 20.6, ash, 4.5 and others, 11.9, including 5.2 of soluble sugars); 50OP - glucan, 30.0 xylan, 17.4; arabinan; 2.8, acetyl groups, 4.0; Klason lignin 22.0, ash, 4.3 and others, 14.1, including 6.4 soluble sugars.

4.2.2 Pretreatment

The mixtures 50ER, 50WS and 50OP were prepared immediately before each pretreatment. The pretreatment was hydrothermal (autohydrolysis) performed in a 600-mL stainless steel reactor model 4842 (Parr Instruments Company, Moline, Illinois, USA) at 220ºC with a liquid-to-solid ratio of 7/1 ($g.g^{-1}$) under non-isothermal conditions, as previously described (Chapter 2). After pretreatment, the liquid and solid fractions were separated using a hydraulic press (Sotel, Portugal) up to 200 Kg/cm^2. The liquid fraction was analyzed and stored at 5ºC until further use. The solid fraction was washed at room temperature with two volumes of water and stored at -10ºC, until further use.

4.2.3 Preparation of hemicellulosic hydrolysates

The liquid fractions, or hemicellulosic hydrolysates, obtained from autohydrolysis of the 50ER, 50WS and 50OP were concentred by evaporation at 50ºC and 40 mbar in order to reduce around 50% of the initial volume (two-fold concentration). The concentrated hemicellulosic hydrolysates were divided in two fractions, one was directly detoxified resulting in a detoxified hemicellulosic hydrolysate from autohydrolysis (AHH), and the other was submitted to post-hydrolysis with sulphuric acid (1% w/w) before detoxification, resulting in detoxified hemicellulosic hydrolysate from post-hydrolysis (PHH).

A combined overliming/active charcoal detoxification process was applied according to the previously reported (Villarreal et al., 2006), with a few modifications. In short, the pH was first adjusted until 8.5 with CaO (powder), and then to 1.8 with sulphuric acid (96%). After, the hemicellulosic hydrolysate was mixed with active charcoal (100-400 mesh) at 5% (w/v) for 1 h, at 30ºC, under stirring at 200 rpm. Finally, the pH was corrected to 5.5 with NaOH (pellets). At the end of each step, the precipitate was removed by filtration through Whatman no. 41 filter paper. The AHH and PHH were sterilized using 0.22-µm membrane filters. The detoxified hemicellulosic hydrolysates were used for inoculum adaptation and in SHCF and SSCF processes.

4.2.4 Microorganism and inoculum preparation

The recombinant xylose-fermenting *S. cerevisiae* BH42 (PYCC 5764) was obtained from the Portuguese Yeast Culture Collection (PYCC). The strains was grown on yeast-malt agar (YMA) plates at 30ºC for 24 hours, stored at 4ºC and renewed periodically in the same medium. The pre-inoculum was prepared in liquid medium containing YNB (6.7 g/L) and glucose (20 g/L) at 35°C and 140 rpm. After 18 h, the cells were harvested by centrifugation (8,500 xg for 10 min) and transferred to fresh liquid medium or hemicellulosic hydrolysate for inoculum adaptation before SH(C)F and SS(C)F experiments.

The inoculum adaptation was performed in 50%-diluted AHH from pretreated 50ER, 50WS or 50OP, and supplemented with yeast extract (1 g/L), $(NH_4)_2HPO_4$ (0.5 g/L), $MgSO_4.7H_2O$ (0.025g/L), glucose (40 g/L) and phthalate buffer (50 mM) at pH 5.5. Yeast was added to 1 g(CDW)/L and incubated at 35ºC, 140 rpm, for 24 h.

4.2.5 Enzymatic hydrolysis

The enzymatic hydrolysis of the solid fraction of pretreated 50ER, 50WS and 50OP was performed at pH 5.5 (phthalate buffer 50 mM) with 20% (w/v) solid loading in the absence and in the presence of AHH. An enzyme dosage of 20.24 FPU/g_{solids} and 60.1 U β-glucosidase/g_{solid} was applied, corresponding to a formulation of two commercial enzyme preparations, Celluclast 1.5L (0.569 g/g_{solid}) and Novozym 188 (0.063 g/g_{solid}), in the dosage predicted by the model for enzymatic hydrolysis previous developed (Chapter 3), according to the fixed variables 20% (w/v) solids and pretreatment at 220ºC. This condition is very close to that predicted for maximal glucose recovery at 20% (w/v) solids (224ºC, 20.16 FPU/g_{solids}). The flasks were incubated in an orbital shaker (ERTAL HT), at 35ºC, 140 rpm, for 144 h. The assays were performed in duplicate.

4.2.6 Separate hydrolysis and (co)-fermentation (SH(C)F) and simultaneous saccharification and (co)-fermentation (SS(C)F)

Fig. 4.1 illustrates the experiments performed under different configurations. The assays were carried out under (non-strictly) anaerobic conditions, more specifically, before incubation the 50-mL Erlenmeyer flasks were sealed with rubber stoppers pierce with needle to liberate CO_2 and sparged with nitrogen to remove air. The 20% (w/v) solid content was obtained by diluting the pretreated solids in deionized water, xylose 10 g/L or hemicellulosic hydrolysate (AHH or PHH). The medium were supplemented as described above for inoculum preparation and the pH was adjusted to 5.5 with phthalate buffer (50 mM). The medium was inoculated with an initial *S. cerevisiae* of 3g(CDW)/L.

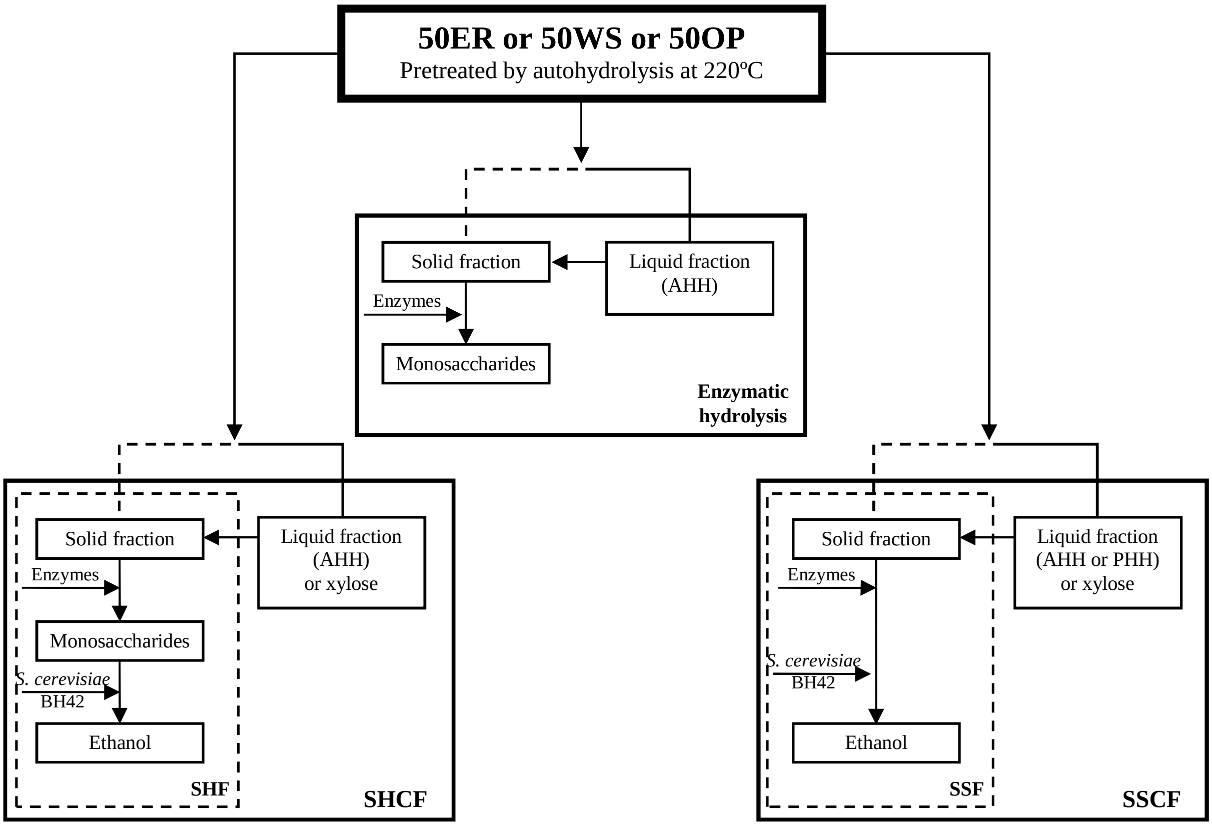

Figure 4.1 Outline of the performed experiments.

The SHCF and SHF experiments were initiated as described above for enzymatic hydrolysis, but incubated at 50ºC for 48 h. Then the flasks were cooled down up to 35ºC, inoculated and incubated at the later temperature, 140 rpm, for 96 h. The SSCF and SSF experiments were initiated with simultaneous enzyme and yeast loading and incubated at 35ºC, 140 rpm, for 96 h. The assays were performed in duplicate. Samples were collected periodically and the concentration of glucose, xylose, xylitol, glycerol, acetic acid and ethanol were quantified by HPLC.

4.2.7 Analytical methods

The solid and liquid (hemicellulosic hydrolysates and post-hydrolysates) fractions obtained after pretreatment and used as substrates for enzymatic hydrolysis and fermentation were characterized following the procedures previously described (Chapter 2). The individual components of substrates and products of enzymatic hydrolysis and fermentation processes were quantified in a high-performance liquid chromatography (HPLC) system (Agilent 1100 Series, Germany) equipped with refractive index (IR) and diode array (DAD) detectors and an Aminex HPX-87H column (Bio-Rad, CA, USA). The column temperature was set to 50°C and the mobile phase of 5 mM H_2SO_4 was used. The flow rate and sample injection volumes were set at 0.4 mL/min and 20 μL, respectively, for the characterization of substrates and at 0.6 mL/min and 5 μL, respectively, for the analysis of products of enzymatic hydrolysis and fermentation. All samples were filtered through 0.22 μm membranes before analysis. Cell growth in the inoculum was evaluated by measuring the absorbance at 640 nm and by cell dry weight (CDW).

4.3 Results and Discussion

In previous work, the ability of processing ER, WS, OP and their mixtures (50ER, 50WS and 50OP) through hydrothermal pretreatment, followed by acid post-hydrolysis of the liquid fraction and enzymatic hydrolysis of the solid fraction, was demonstrated (Chapters 2 and 3). This work, aimed at processing these lignocellulosic mixtures for ethanol production combining hydrothermal pretreatment (autohydrolysis), enzymatic hydrolysis at high solid loading (20% w/v) and co-fermentation of glucose and xylose (the later from hemicellulosic hydrolysates, with or without post-hydrolysis), through SHCF and SSCF, employing commercial enzymes and the recombinant xylose-fermenting *S. cerevisiae* BH42.

4.3.1 Composition of the resulting solid and liquid fractions recovered after pretreatment

Previous studies on hydrothermal pretreatment of the three materials and their mixtures (Chapter 2) and on the combination of pretreatment and enzymatic hydrolysis for maximal glucose recovery (Chapter 3), revealed that a compromise between high sugar recovery in the hemicellulosic hydrolysate (liquid fraction) and high cellulose digestibility (solid fraction) needs to be achieved. High pretreatment temperature (e.g. 230ºC non-isothermal) usually contributes to maximal cellulose digestibility, but generates high amounts of sugars degradation products in the hemicellulosic hydrolysate, with consequent low sugar (xylose) recovery yield and high content of inhibitory compounds in this fraction (Chapter 2). The hydrothermal pretreatment performed at 220ºC still leads to high cellulose digestibility with the three feedstocks (>80%) (Chapter 3), maintains xylose equivalents (XOS and xylose) over 15 g/L, and keeps inhibitory compounds at moderate levels (e.g. furfural and acetic acid at approx. 2 and 5 g/L, respectively) (data not shown).

The hemicellulosic hydrolysates recovered after autohydrolysis and post-hydrolysis were concentrated (2-fold) and detoxified in order to simulate sugar (xylose) content in pretreatment at higher solid loading and to allow fermentation with the available recombinant xylose-fermenting *S. cerevisiae*. The composition of both cellulo-lignin (solid fraction) and the hemicellulosic hydrolysates from autohydrolysis (AHH) and from further post-hydrolysis (PHH) are depicted in Table 4.1. The concentration step, which might also be applied by industry to concentrate cellulosic and hemicellulosic hydrolysates, significantly reduces volatile toxic compounds, such as furfural, which concentration was decreased in one order of magnitude, i.e. in approx. 90%. This observation was also reported by other authors (Carvalheiro et al., 2005; Villarreal et al., 2006). The combination of evaporation and detoxification has partially removed acetic acid, up to 25%.

As expected, XOS were the product found at higher concentrations (18-24 g/L) in the AHH, confirming the efficiency of autohydrolysis in the solubilization of the hemicellulosic fraction. GlcOS were also found at significant concentrations (10-12 g/L), which is probably correlated with the presence of starch and others non-structural soluble sugars in the feedstocks. In fact, WS and OP contain significant amounts of starch and OP of non-structural soluble saccharides (Cara et al., 2012; Chapter 2 – Part A). AOS were virtually absent as the pretreatment severity was enough to hydrolyse the OS into arabinose. The concentrations of total monosaccharides in AHH were similar for all mixtures (18-22 g/L), with xylose at 12-14 g/L and glucose and arabinose at 3-4 g/L. While 50WS contained more arabinose, from arabinoxylans of WS, 50OP contained more glucose as denoted by the presence of soluble glucose in OP composition (Ballesteros et al., 2011; Cara et al., 2008; Chapter 2). Organic acids (formic adding acetic) were found in the range of 9 g/L. Furans (furfufal and HMF) resulting from thermic degradation of pentoses and hexoses (Palmqvist and Hahn-Hägerdal, 2000) were detected at very low concentrations (<0.5 g/L), due to the concentration/detoxification process.

With the post-hydrolysis process applied after autohydrolysis, the OS were converted into monosaccharides. Accordingly, the PHH revealed substantial higher xylose (32-33 g/L) and glucose (10-12 g/L) concentrations. Acetic acid concentration was also increased after post-hydrolysis due the extensive hydrolysis of hemicellulose oligomers. The acetyl groups linked to XOS are released at more severe processing of hemicellulosic components, as previously observed with *Eucalyptus globulus* wood (Garrote et al., 2001). Furfural concentration slight increased after chemical post-hydrolysis, due the labile nature of pentoses at high temperature. HMF concentration remained nearly constant, which was correlated with the low degradation of hexoses. As levulinic acid results from extensive degradation of hexoses, in this work it was only detected after post-hydrolysis and at low concentrations.

Table 4.1 Composition of the hemicellulosic hydrolysates (liquid fraction) recovered after autohydrolysis and chemical post-hydrolysis (when applied) followed by concentration and detoxification (AHH and PHH, respectively), and respective cellulo-lignin (solid fraction) of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) (50ER, 50WS and 50OP).

Fraction	Component	Feedstock mixture					
		50ER		50WS		5OP0	
		AHH[a]	PHH[b]	AHH	PHH	AHH	PHH
Liquid fraction (g/L)	Glucose	3.36	10.42	3.27	10.86	4.23	12.25
	GlcOS[c]	10.41	nd[f]	11.37	nd	11.56	nd
	Xylose	11.66	32.20	12.08	33.38	13.53	32.49
	XOS[d]	18.44	nd	24.09	nd	17.89	nd
	Arabinose	3.22	3.63	4.13	3.58	3.78	3.95
	AOS[e]	0.00	nd	0.00	nd	0.00	nd
	Formic acid	3.31	3.41	3.33	3.12	3.39	3.22
	Acetic acid	5.73	8.85	5.78	7.99	5.49	8.09
	Levulinic acid	0.00	0.30	0.00	0.27	0.00	0.30
	Furfural	0.02	0.16	0.01	0.24	0.01	0.23
	HMF	0.39	0.37	0.36	0.30	0.49	0.45
	Phenols[g]	1.55	1.38	2.01	1.24	2.44	1.37
Solid fraction (g/100g)	Cellulose	50.00		50.71		49.31	
	Hemicellulose	4.34		5.03		5.36	
	Lignin	38.99		37.05		40.93	
	Others	6.67		7.21		7.84	

[a] hemicellulosic hydrolysate obtained after autohydrolysis, concentration and detoxification; [b] hemicellulosic hydrolysate obtained after autohydrolysis followed by post-hydrolysis, concentration and detoxification; [c] glucooligosaccharides [d] xylooligosaccharides; [e] arabinooligosaccharides; [f] not determined; [g] total phenolic compounds.

The pretreatment conditions (autohydrolysis at 220ºC, non-isothermal) applied to 50ER, 50WS and 50OP promoted an extensive solubilization of hemicellulose, remaining only 4-5 (g/100g dry matter) in the solid fraction. Consequently, the solid fraction was enriched in cellulose and lignin to contents of 49-51 and 37-41 (g/100g dry matter), respectively. The cellulose was enriched in 17-19% in comparison to original materials. These results are in agreement to those obtained in the previous study of autohydrolysis with the same feedstock mixtures (Chapter 2 – Part B). Similar cellulose (41.9-45.2%) and acid-insoluble residues (43.6-50.4%) were found in olive tree pruning pretreated solids after steam-explosion at 220ºC (Cara et al., 2008). In a study of eucalyptus wood autohydrolysis, similar cellulose content (50.9%) and lower lignin content (24.3%) were found in pretreated solids, but milder conditions were applied (195ºC) (Garrote et al., 1999). In fact, lower pretreatment temperatures lead to higher hemicellulose content in the solid fraction (Chapter 2).

4.3.2 Effect of hemicellulosic hydrolysates on the enzymatic hydrolysis of pretreated solids

The enzymatic hydrolysis of the feedstock mixtures 50ER, 50WS and 50OP was performed at 35ºC, with 20% (w/v) solids obtained from pretreatment at 220ºC and an enzyme dosage of 20.24 FPU/g_{solids}, in presence or absence of the respective hemicellulosic hydrolysates (AHH) (Fig. 4.2). For the three different mixtures, a similar profile of enzymatic hydrolysis was observed. Glucose concentrations of 83-85 g/L were achieved after 144 h of enzymatic hydrolysis in presence or absence of hemicellulosic hydrolysates (AHH). However, the enzymatic yield was different since, in addition to the potential glucose from cellulose hydrolysis, there is the glucose from the GlcOS present in the hemicellulosic hydrolysate (AHH). Thus, a decrease of 5.7, 8.0 and 13.2% in enzymatic hydrolysis yield of 50ER, 50WS and 50OP, respectively, was observed in presence of hemicellulosic hydrolysates (AHH). In fact, the compounds formed during the pretreatment, namely furans, organic acids and phenolic compounds can inhibit the cellulolytic enzymes used (Viikari et al., 2012). However, under the conditions applied, the concentration of these compounds had a low influence in the cellulolytic enzymes. The presence of soluble saccharides (glucose, xylose, GlcOS and XOS) in the hemicellulosic hydrolysates may lead to competitive inhibition (Baumann et al., 2011; Modenbach and Nokes, 2013; Teugjas and Väljamäe, 2013), which was observed to be more pronounced at 35ºC than at 50ºC (Teugjas and Väljamäe, 2013). The lower enzymatic hydrolysis rate obtained in this work, comparing with the previous studies under similar conditions (Chapter 2 – Part A and Chapter 3), is justified by the lower temperature applied in this enzymatic hydrolysis process, 35ºC, which is the temperature used in the following SS(C)F experiments and in the fermentation step of SH(C)F experiments.

An increase of xylose concentration was observed during the enzymatic hydrolysis process in the presence of hemicellulosic hydrolysates (AHH) (Fig. 4.2), which confirmed that the enzymes formulation used in this work can promote the hydrolysis of XOS obtained during the pretreatment process, as Celluclast 1.5L and Novozym 188 contain xylanolytic enzymes (Van Dyk and Pletschke, 2012; Wyman et al., 2004). The highest xylose concentrations obtained were in the range of 10-11 g/L at 144 h of enzymatic hydrolysis. These xylose concentrations are approx. 25% lower to those of initial xylose in PHH (around 14 g/L), suggesting that hydrolysis of XOS can be performed by enzymes in simultaneous with cellulose and GlcOS hydrolysis, but are less effective than acid post-hydrolysis towards xylose production.

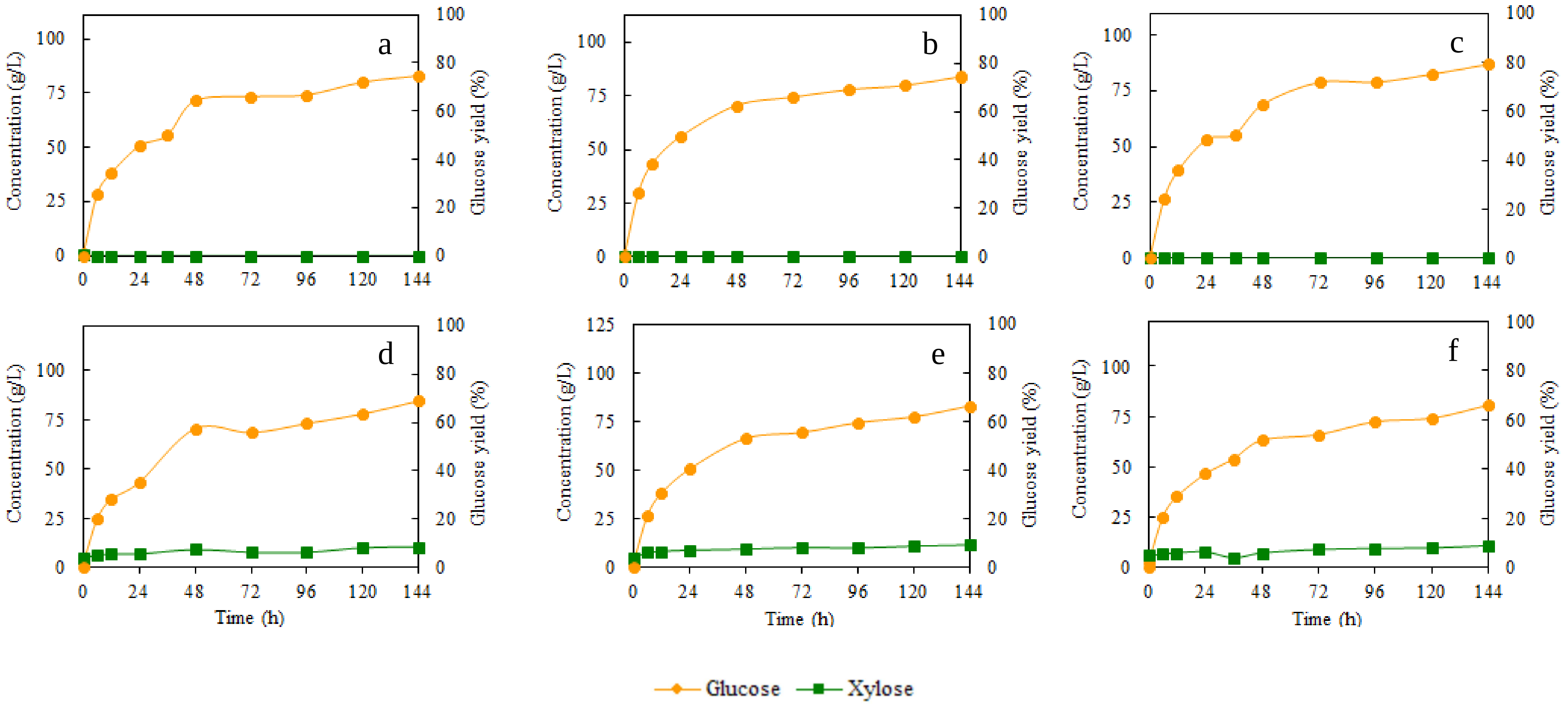

Figure 4.2 Kinetic profile of the enzymatic hydrolysis of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP), 50ER (a, d), 50WS (b, e) or 50OP (c, f), in absence (a, b, c) or presence (d, e, f) of hemicellulosic hydrolysates (AHH). Enzymatic hydrolysis was performed with 20% (w/v) of solids obtained from pretreatment (autohydrolysis) at 220ºC and an enzyme dosage of 20.24 FPU/g$_{solids}$, at 35ºC, pH 5.5, 140 rpm.

4.3.3 SHF and SSF of pretreated solids from different mixtures of ER, WS and OP

SHF and SSF experiments combined pretreated feedstock mixtures (50ER, 50WS and 50OP) at 20% w/v solids with enzymes (at 20.24 FPU/g_{solids}) and the yeast *S. cerevisiae* BH42 (at 3 g(CDW)/L) (Fig. 4.3). Compared to SSF, SHF process had a prior enzymatic hydrolysis period of 48 h at 50ºC, before yeast loading. The fermentation step of SHF and the SSF process were performed at 35ºC for 96 h. Therefore, the main difference observed between the two processes was that, in SHF process, the fermentation step was initiated with a glucose concentration of 65-70 g/L, while in the SSF process, maximum glucose concentration was detected after 6 hours at approx. 10 g/L.

Consequently, the higher glucose available in SHF, in comparison to SSF, promoted higher ethanol productivity, markedly observed in the first 12-h fermentation period (Fig. 4.3, Table 4.2). After 24-h fermentation, the ethanol concentration reached a plateau in the range of 35-41 g/L in SHF with all feedstock mixtures.

On the other hand, in SSF, the accumulation of glucose in the medium during the first 6 h, resulted from higher rate of cellulose enzymatic hydrolysis than glucose consumption by yeast, which conducted to similar ethanol productivity in both processes during the same period (6h) (Fig. 4.3). However, after 12-h fermentation, the ethanol productivity was lower in SSF than in SHF (Table 4.2), due to the limitation of glucose consumption rate of *S. cerevisiae* BH42 by enzymatic hydrolysis. Accordingly, the ethanol concentration reached a plateau in the range of 35-40 g/L only after 48 h in SSF with all feedstock mixtures. Ethanol productivity was clearly lower in SSF than in SHF (difference higher than 10%), but the difference in final ethanol concentration and yields was smaller (lower than 10%). Among all, the less efficient process was the SSF of 50OP, reaching only 37 g/L and an overall yield of 0.31 g/g (total sugars). In fact, residual monosaccharides, approx. 5 g/L of glucose and 3 g/L xylose, were detected in the medium after 96 h. OP was previously described as containing hydroxytyrosol, tyrosol, and oleuropein, which are phenolic compounds with high antimicrobial activity (Fernández-Bolaños et al., 2012; Medina et al., 2013). These phenolic compounds might be attached to the solids and released to the medium during enzymatic hydrolysis, increasing the toxic level of the mixture with higher OP content during the SSF process.

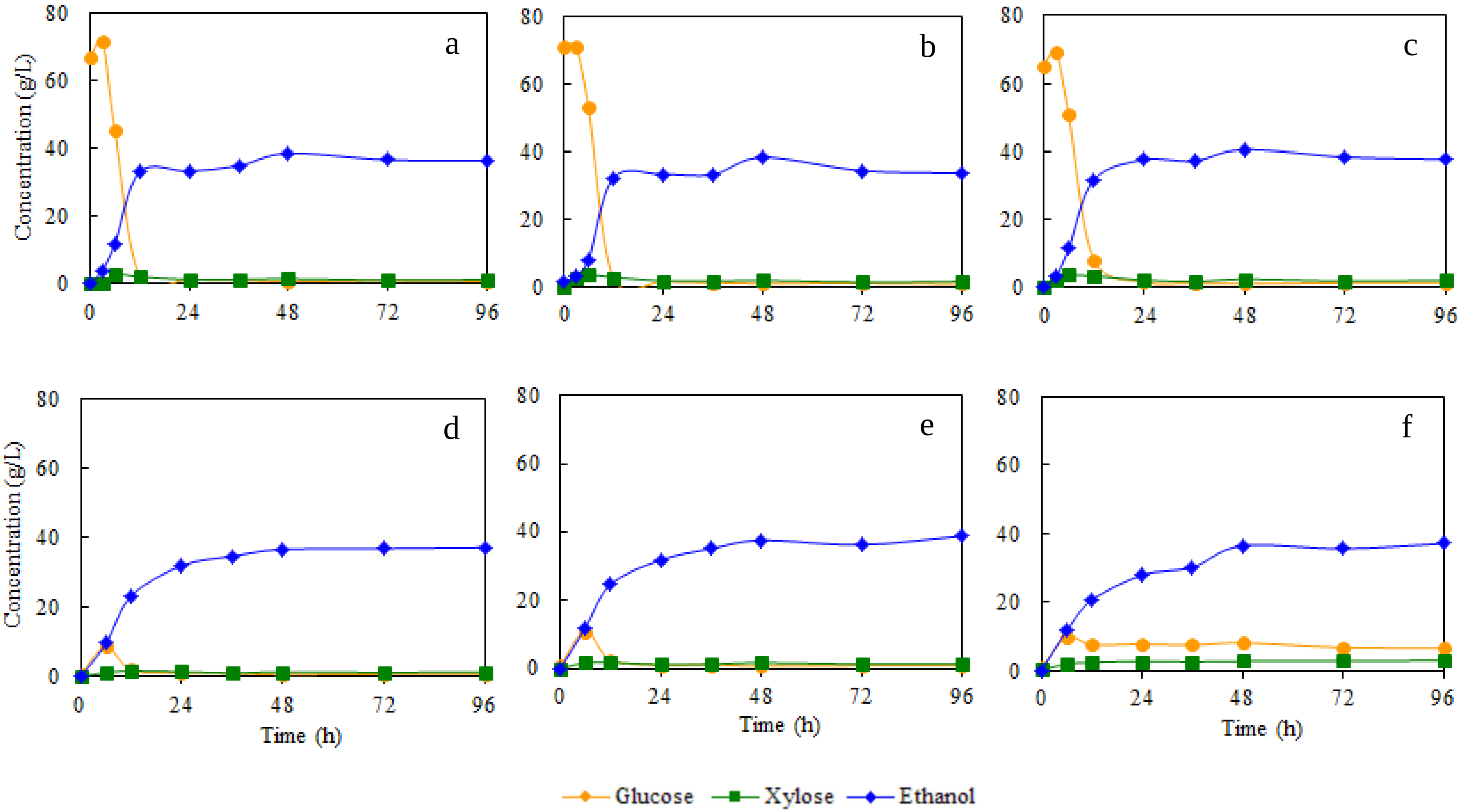

Figure 4.3 SHF (a, b, c) and SSF (d, e, f) of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP), 50ER (a, d), 50WS (b, e) or 50OP (c, f). The processes were performed with 20% (w/v) of solids obtained from pretreatment at 220ºC, an enzyme dosage of 20.24 FPU/g_{solids} and the yeast *Saccharomyces cerevisiae* BH42 (3 g(CDW)/L), at pH 5.5. The SHF was initiated with enzyme loading and incubated for 48 h at 50ºC, 140 rpm, followed by yeast inoculum and incubation under anaerobiosis for 96 h at 35ºC, 140 rpm. In the SSF, enzyme and yeast were simultaneously loaded, followed by incubation under anaerobiosis for 96 h at 35ºC, 140 rpm.

Table 4.2 **Fermentative parameters and residual xylose (after 96 h of fermentation) of SH(C)F and SS(C)F of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP) (50ER, 50WS and 50OP). The processes were performed with 20% (w/v) of solids obtained from pretreatment at 220ºC, an enzyme dosage of 20.24 FPU/g_{solids} and the yeast *Saccharomyces cerevisiae* BH42 (3 g(CDW)/L) at pH 5.5. The SH(C)F was initiated with enzyme loading and incubated for 48 h at 50ºC, 140 rpm, followed by yeast inoculum and incubation under anaerobiosis for 96 h at 35ºC, 140 rpm. In the SS(C)F, enzyme and yeast were simultaneously loaded, followed by incubation under anaerobiosis for 96 h at 35ºC, 140 rpm.**

		SHF	**SSF**	**SHCF$_{xylose}$**	**SSCF$_{xylose}$**	**SHCF$_{AHH}$**	**SSCF$_{AHH}$**	**SSCF$_{PHH}$**
50ER	**Ethanol (g/L)**	40.85±3.06	38.59±1.34	40.85±0.03	41.01±0.29	41.47±1.07	39.05±1.55	40.42±0.57
	Yp/s (g/g)	0.35±0.03	0.33±0.01	0.32±0.00	0.32±0.00	0.25±0.01	0.23±0.01	0.25±0.00
	Qp (g/L.h) (12h)	2.82±0.07	1.97±0.03	2.74±0.06	1.46±0.42	2.69±0.01	1.74±0.06	1.87±0.02
	Xylose (g/L)	1.34±0.03	1.38±0.08	4.69±0.13	5.30±1.20	6.97±0.08	6.39±0.15	5.96±0.12
50WS	**Ethanol (g/L)**	40.96±3.59	40.03±1.76	43.72±1.41	42.22±1.05	40.71±0.77	37.18±1.71	44.06±0.50
	Yp/s (g/g)	0.34±0.03	0.33±0.01	0.33±0.01	0.32±0.01	0.23±0.00	0.21±0.01	0.27±0.00
	Qp (g/L.h) (12h)	2.75±0.08	2.10±0.06	2.75±0.10	1.98±0.06	2.67±0.02	1.58±0.47	2.11±0.04
	Xylose (g/L)	1.63±0.02	1.43±0.08	5.39±0.09	3.74±0.08	8.53±0.20	7.17±0.09	7.04±0.32
50OP	**Ethanol (g/L)**	40.83±0.31	37.16±1.02	41.86±2.08	37.09±0.30	40.08±0.28	29.15±0.65	35.65±2.19
	Yp/s (g/g)	0.34±0.00	0.31±0.01	0.33±0.02	0.29±0.00	0.24±0.00	0.17±0.00	0.22±0.01
	Qp (g/L.h) (12h)	2.09±0.79	1.80±0.11	2.06±0.73	1.68±0.06	2.27±0.03	1.61±0.00	1.82±0.06
	Xylose (g/L)	2.11±0.07	2.62± 0.31	6.41±0.04	7.64±0.26	8.58±0.34	8.27±0.37	8.49±0.54

AHH - hemicellulosic hydrolysate obtained after autohydrolysis, concentration and detoxification; PHH - hemicellulosic hydrolysate obtained after autohydrolysis followed by post-hydrolysis, concentration and detoxification;

4.3.4 SHCF and SSCF processes for bioethanol production from different mixtures of ER, WS and OP

First, the influence of the presence of xylose (10 g/L) in SHCF and SSCF processes was evaluated under the same conditions of SHF and SSF (Fig. 4.4, Table 4.2), This approach allowed monitoring the effect of both process configurations on glucose and xylose co-fermentation in the absence of inhibitor compounds from hemicellulosic hydrolysates.

The profiles observed in these SHCF and SSCF experiments were very similar to those obtained in SHF and SSF, respectively, namely with respect to cellulose hydrolysis, glucose consumption and ethanol production. In general, ethanol yield and productivities were slightly lower than those observed in SHF and SSF (Table 4.2), but SHCF and SSCF experiments with xylose, in general, led to slightly higher ethanol concentrations (Table 4.2), suggesting that ethanol is being produced from xylose, but at lower yields. In fact, *S. cerevisiae* BH42 is described to have lower ethanol yields from xylose (0.28 g/g) than from glucose (0.43 g/g) in defined medium (Sonderegger et al., 2004).

In the SHCF experiments, glucose concentration obtained after 48 h of enzymatic hydrolysis resulted in 66-68 g/L. The glucose consumption, had a similar profile for the three feedstock mixtures (50ER, 50WS and 50OP) and, after 12 h, almost all glucose in the medium was consumed. Xylose consumption was observed in all cases but, after 48 hours it virtually stopped, remaining approx. 30% of initial xylose. Under SHCF the higher xylose consumption rate was observed between 12 and 36 h of fermentation, immediately after glucose depletion. This is due to the competitive nature of glucose and xylose transport, where Hxts transporters have much higher preference for glucose (Leandro et al., 2009). In SHCF, similar ethanol concentrations were achieved with the different feedstock mixtures, reaching 41-44 g/L, but after 12 h the concentration was already 30-40 g/L.

In SSCF experiments, the accumulation of glucose in the medium was observed during the first 6 h as in the SSF experiments (see Fig. 4.3), with similar ethanol productivity to that of SHCF the same period (6h) (Fig. 4.4). However, after 12 h fermentation, the ethanol productivity was lower in SSCF than in SHCF (Fig. 4.4 and Table 4.2), reaching only 20-30 g/L at that time. The ethanol concentration reached a plateau in the range of 35-44 g/L only after 48 h in SSCF with all feedstock mixtures (Fig. 4.4, Table 4.2). In opposite to the observed in SHCF, the higher xylose consumption rate in SSCF experiments was observed in the first 12 h. A similar behaviour was previously reported in SSCF with WS using recombinant xylose-fermenting *S. cerevisiae* strains, where a high initial xylose concentration and a low glucose concentration minimized the inhibition of xylose transport by glucose, favouring co-consumption (Fonseca et al., 2011; Olofsson et al., 2008). Even though, xylose was not completely consumed, probably because glucose was prematurely exhausted from the culture medium. In fact, xylose fermentation is enhanced by low (but non-zero) glucose concentration, not only due to relieve of transport inhibition, but also to activation of the enzymes of the lower glycolytic pathway and to improved co-factor regeneration (Boles et al., 1996; Pitkänen et al., 2003). In the case of 50WS only 20% of initial xylose remained in the medium. In the case of SSCF with 50OP, and similarly to the SSF with 50OP, the final ethanol concentration, the ethanol yield and productivity were lower than for other mixtures or even than for the same mixture under SHCF. In this case, a significant amount of residual monosaccharides, approx. 9 g/L of glucose and 8 g/L xylose, were detected in the medium after 96 h (Fig. 4.4, Table 4.2). This residual xylose concentration represented approx. 40% of initial xylose.

These preliminary SHCF and SSCF with xylose were performed to further understand the use of complex hemicellulosic hydrolysates for glucose and xylose co-consumption and increased ethanol final concentrations in comparison to SHF and SSF using only the cellulose fraction. Once the glucose and xylose co-fermentation process has been demonstrated, even though xylose was not completely and efficiently fermented, the performance of SHCF and SSCF process using hemicellulosic hydrolysate from pretreated mixtures of ER, WS and OP was performed to evaluate the feasibility of converting the whole slurry of the different mixtures (50ER, 50WS and 50OP) into ethanol. The hemicellulosic hydrolysate recovered after autohydrolysis (AHH) of the three feedstock mixtures, rich in OS, was used in the SHCF and SSCF, as the commercial enzyme preparations used in this work have xylanolytic activities. Moreover, the hemicellulosic hydrolysate recovered after acid post-hydrolysis (PHH) was also used, since the XOS obtained in autohydrolysis are here completely hydrolysed to xylose (see Table 4.1). This approach envisaged to have high initial xylose level for SSCF experiments once the low glucose and the high xylose concentrations favours their co-transport and consequent their co-fermentation (Fonseca et al., 2011; Öhgren et al., 2006; Olofsson et al., 2008).

Therefore, three process configurations for enzymatic hydrolysis and co-fermentation of feedstock mixtures have been assessed: i) SHCF with AHH (SHCF_{AHH}); ii) SSCF with AHH (SSCF_{AHH}); SSCF with PHH (SSCF_{PHH}) (Fig. 4.5).

In the SHCF_{AHH} experiments the profile of glucose and xylose consumption was similar with the different mixtures and comparable to the observed in the previous experiment using xylose in the liquid medium (Fig. 4.4). The glucose produced during enzymatic hydrolysis (63-72 g/L) was consumed in the first 24 h for all mixtures. However, while in SHCF_{AHH} of 50ER and 50WS the glucose concentration was already below 10 g/L at 12 h, in SHCF_{AHH} of 50OP, glucose was still approx. 20 g/L, suggesting that there are components specifically from OP that might inhibit yeast metabolism, as phenolic compounds (see Table 4.1). Anyway, ethanol productivities were similar to those obtained in SHF and SHCF with xylose (see Table 4.2) and maximum ethanol concentrations were above 40 g/L in all SHCF_{AHH}, including with 50OP. Therefore, ethanol yield was in the range of 0.23-0.25 g/g in SHCF_{AHH} against 0.34-0.35 g/g in SHF and 0.32-0.33 in SHCF with xylose. The reduction in yield was expected due to the intrinsic lower ethanol yield from xylose than from glucose, as determined in defined medium (Sonderegger et al., 2004) and due to the inhibition of xylose transport by glucose in *S. cerevisiae* BH42 (Fonseca et al., 2011).

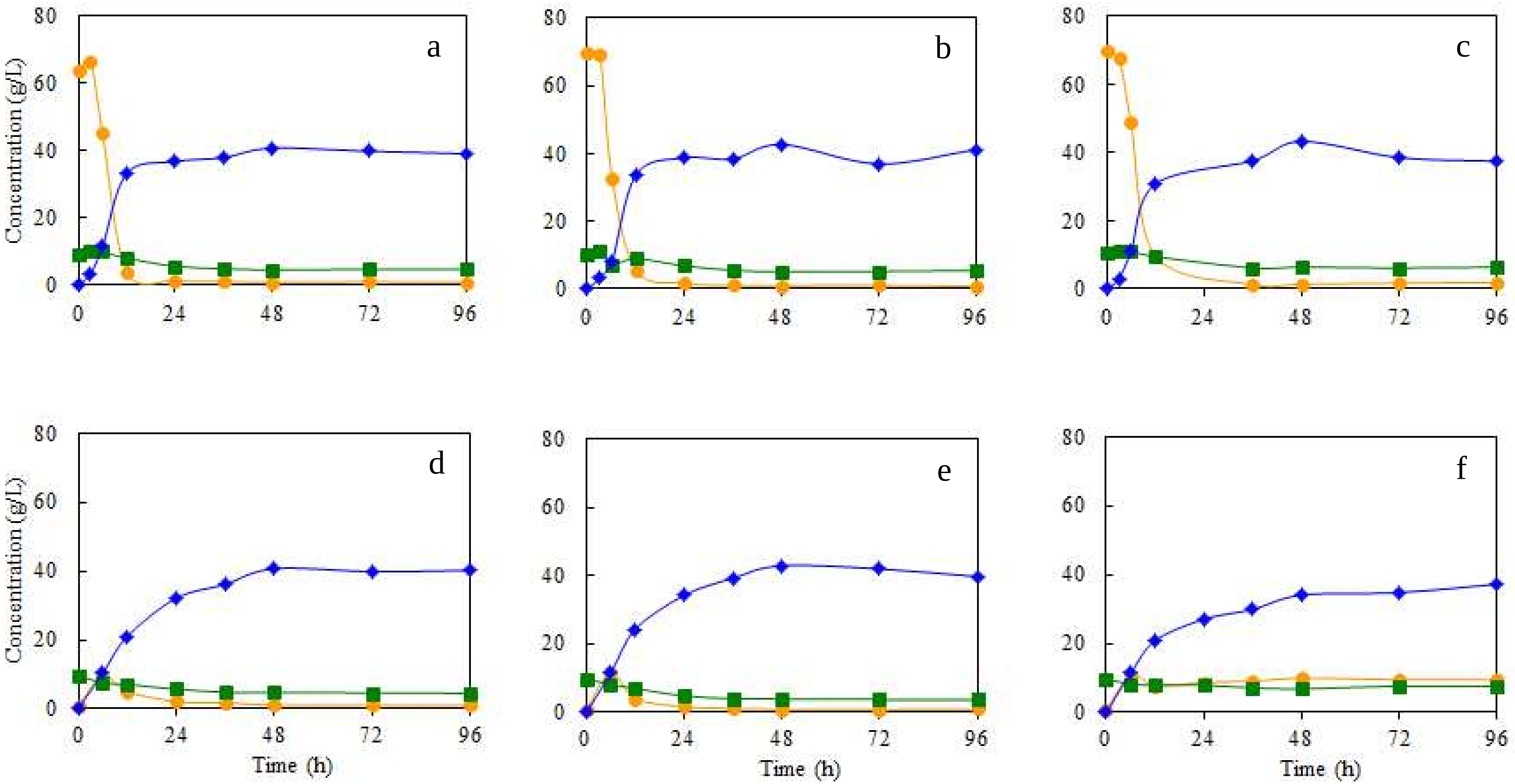

Figure 4.4 SHCF (a, b, c) and SSCF (d, e, f) of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP), 50ER (a, d), 50WS (b, e) or 50OP (c, f) in the presence of xylose (10 g/L). The processes were performed with 20% (w/v) of solids obtained from pretreatment at 220ºC, an enzyme dosage of 20.24 FPU/g_{solids} and the yeast *Saccharomyces cerevisiae* BH42 (3 g(CDW)/L), at pH 5.5, in the presence of xylose (10 g/L). The SHCF was initiated with enzyme loading and incubated for 48 h at 50ºC, 140 rpm, followed by yeast inoculum and incubation under anaerobiosis for 96 h at 35ºC, 140 rpm. In the SSCF, enzyme and yeast were simultaneously loaded, followed by incubation under anaerobiosis for 96 h at 35ºC, 140 rpm.

The $SSCF_{AHH}$ of 50ER, 50WS and 50OP was the process configuration showing the lowest final ethanol concentrations and yields (based on total sugars), reaching 39 g/L and 0.23 g/g with 50ER, 37 g/L and 0.21 g/g with 50WS and only 29 g/L and 0.17 g/g with 50OP, respectively. In line with these results is the accumulation of glucose in all $SSCF_{AHH}$ processes, up to 8 g/L in 50ER and 50WS and 19 g/L in OP after 96 h of fermentation. Interestingly, xylose accumulation in $SSCF_{AHH}$ is slightly lower than in $SHCF_{AHH}$, suggesting a more efficient co-consumption of xylose in the presence of lower glucose concentrations. In sum, the $SSCF_{AHH}$ followed the trends of previous SS(C)F experiments (see Fig. 4.3 and 4.4, Table 4.2), but the presence of phenolic compound and other inhibitors (see Table 4.1), like acids (acetic and formic) and furans (here mainly HMF), may have contributed to less efficient fermentation processes. The effect of these compounds was apparently less pronounced in $SHCF_{AHH}$ probably because higher glucose concentrations may have contributed to higher yeast fitness and to a faster conversion process. Even though the adaptation of inoculum of *S. cerevisiae* BH42 was performed in 50% hemicellulosic hydrolysate (AHH) before fermentation, the performance of the yeast was limited in $SSCF_{AHH}$. Probably, a longer period of adaptation under higher hydrolysate concentrations or a higher inoculum loading might have conducted to better performance.

In $SSCF_{PHH}$ the bioconversion process revealed a better performance than the $SSCF_{AHH}$ (Fig. 4.5, Table 4.2). In fact, the glucose profile followed those of the previous processes of SSF and of SSCF with xylose (see Fig. 4.3 and 4.4), rather than the ones of $SSCF_{AHH}$. First, glucose accumulated in the first 6 hours up to approx. 10 g/L. Then, with 50ER and 50WS, it was maintained below 5 g/L till the end of the fermentation process. However, with 50OP, glucose accumulated up to approx. 14 g/L. Interestingly, effective xylose co-consumption with glucose was observed since the initial xylose concentration of about 14 g/L in $SSCF_{PHH}$ decreased to about half (6-8 g/L) within 24 h. This higher xylose consumption observed in $SSCF_{PHH}$ in comparison with $SSCF_{AHH}$ and $SHCF_{AHH}$ is confirmed by lower residual xylose in the former configuration after 96 h (Table 4.2), inasmuch XOS were certainly fully hydrolysed to xylose, at approx. 14 g/L (initial concentration) prior to fermentation, while only approx. 10 g/L was obtained in the enzymatic hydrolysis step of $SHCF_{AHH}$ and approx. 6 g/L were found in the beginning of $SSCF_{AHH}$. Therefore, the presence of xylose in higher concentration than glucose favours their co-consumption (Fonseca et al., 2011; Olofsson et al., 2008). These profiles resulted in final ethanol concentrations and yields (based on total sugars) of 40 g/L and 0.25 g/g with 50ER, 44 g/L and 0.27 g/g with 50WS and 36 g/L and 0.22 g/g with 50OP, respectively, in the $SSCF_{PHH}$ process configuration. As mentioned above, the worst performance obtained with 50OP comparing with the other two mixtures (50ER and 50WS) was probably related to higher concentration of phenolic compounds from olive tree (hydroxytyrosol, tyrosol, and oleuropein), which have been shown to have a great antimicrobial activity (Fernández-Bolaños et al., 2012; Medina et al., 2013). The better performance of $SSCF_{PHH}$ compared to $SSCF_{AHH}$ can be attributed to lower total phenolic compounds concentration. The decrease in the total phenolic concentration after acid

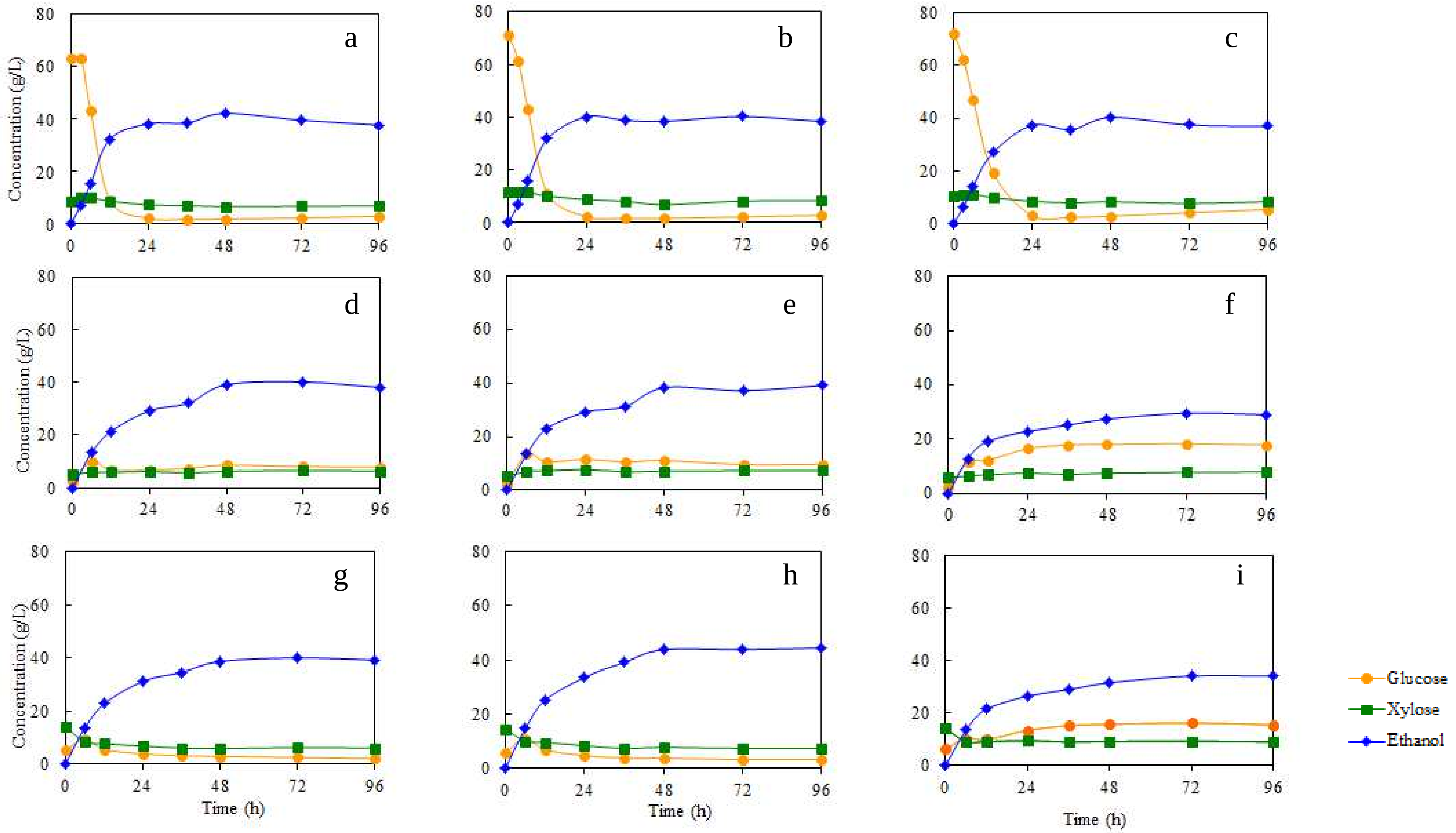

Figure 4.5 SHCF (a, b, c) and SSCF (d, e, f, g, h, i) of different mixtures of eucalyptus residues (ER), wheat straw (WS) and olive tree pruning (OP), 50ER (a, d, g), 50WS (b, e, h) or 50OP (c, f, i) in the presence of hemicellulosic hydrolysate from autohydrolysis (AHH) (a, b, c, d, e, f) or hemicellulosic hydrolysate from post-hydrolysis (PHH) (g, h, i). The processes were performed with 20% (w/v) of solids obtained from pretreatment at 220ºC, an enzyme dosage of 20.24 FPU/g_{solids} and the yeast *Saccharomyces cerevisiae* BH42 (3 g(CDW)/L), at pH 5.5, in the presence of hemicellulosic hydrolysates after concentration/detoxification. The SHCF was initiated with enzyme loading and incubated for 48 h at 50ºC, 140 rpm, followed by yeast inoculum and incubation under anaerobiosis for 96 h at 35ºC, 140 rpm. In the SSCF, enzyme and yeast were simultaneously loaded, followed by incubation under anaerobiosis for 96 h at 35ºC, 140 rpm.

post-hydrolysis was previously reported and was attributed to their selective precipitation at the acidic pH of chemical post-hydrolysis, and subsequent removal by filtration (Duarte et al., 2009).

Fermentation by-products, such as xylitol and glycerol, were found under the different process configurations and for all feedstock mixtures. Xylitol, an undesirable by-product generated by recombinant XR/XDH *S. cerevisiae* strains as a response to the lack of the co-factor NAD^+ under anaerobiosis, was produced in all co-fermentation experiments at concentrations below 2 g/L, except for $SSCF_{AHH}$ and $SSCF_{PHH}$, where it reached 3 g/L. Xylitol cconcentrations in the range of 1.3-4.3 g/L were reported to SSF of pretreated wheat straw (Olofsson et al., 2008). Glycerol was detected in higher concentrations than xylitol, reaching 3-4 g/L in the different process for all feedstock mixture. Glycerol was quantified at 6.7 g/L during SSF of wheat straw using *S. cerevisiae* TMB3400 (Olofsson et al., 2008). Also, processing pretreated wheat straw by SSCF at initial xylose concentrations of approx. 17 g/L, *S. cerevisiae* strains TMB3400 and 34006 generated xylitol and glycerol concentrations of 4-5 g/L each (Fonseca et al., 2011). An increase of acetic acid concentration also was observed throughout time for the three feedstock mixture studied reaching values higher than 6 g/L, which can also contribute to significant inhibitory effects (Palmqvist and Hahn-Hägerdal, 2000).

Comparing the different process configurations, including those with absence of xylose, SHF and SSF, and those of glucose/xylose co-fermentation, SHCF and SSCF, it is possible to verify the contribution of this pentose for ethanol production but, at the same time, the inhibition of *S. cerevisiae* BH42 by toxic compounds from hemicellulosic hydrolysates. This effect was particular evident for SSCF of 50OP, probably due to presence of higher concentration of phenolic compounds derived from olive tree in 50OP hemicellulosic hydrolysates, like hydroxytyrosol, tyrosol, and oleuropein.

4.4 Conclusions

The use of different lignocellulosic feedstock mixtures of ER, WS and OP for 2G bioethanol production was demonstrated under different process configurations. The use of the cellulosic and hemicellulosic fractions obtained after hydrothermal pretreatment of feedstock mixtures, was assessed by SH(C)F and SS(C)F at high solid loading (20% w/v), and generated ethanol concentrations higher than 40 g/L. These concentrations are in the range of those suitable for cost-effective ethanol recovery processes. Several aspects will deserve future research to improve the process of converting mixtures of ER, WS and OP into bioethanol. Those include the operation of high solids at the pretreatment level, which may conduct to higher xylose concentrations in the hemicellulosic hydrolysates and thus contribute to improved xylose co-consumption with glucose, preferentially by SSCF. Also, the reduction of enzyme loading in SSCF may be beneficial to maintain low glucose concentration during a longer fermentation period for maximal xylose fermentation. A similar effect might be obtained by increasing yeast loading. To face the presence of inhibitors (e.g. acids and phenolics) the higher yeast loading and/or more efficient adaptation protocols may improve the bioconversion process. However, the use of more robust and efficient recombinant xylose-fermenting *S. cerevisiae* strains might be required.

Nevertheless, the potential use of different feedstock mixtures, containing ER, WS and OP, in multi-feedstock 2G bioethanol biorefineries located in Southern Europe was demonstrated, with the possibility of using constant operational conditions throughout the year.

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4.6 Supplementary data

Table 4.3 Potential sugar recovery (g/L) from solid and liquid fraction (hemicellulosic hydrolysate) recovered after pretreatment performed at 220ºC, post-hydrolysis (when applied) and concentration/detoxification (when applied) in SH(C)F and SS(C)F processes.

		Solid fraction	Liquid fraction	Total
SSF e SHF				
50ER	Glucose	111.12	0.00	111.12
	Xylose	6.89	0.00	6.89
50WS	Glucose	112.70	0.00	112.70
	Xylose	8.77	0.00	8.77
50OP	Glucose	109.59	0.00	109.59
	Xylose	9.34	0.00	9.34
$\mathrm{SSCF_{AHH}}$ and $\mathrm{SHCF_{AHH}}$				
50ER	Glucose	111.12	14.93	126.04
	Xylose	6.89	32.61	39.50
50WS	Glucose	112.70	15.90	128.60
	Xylose	8.77	39.45	48.22
50OP	Glucose	109.59	17.08	126.67
	Xylose	9.34	33.86	43.20
$\mathrm{SSCF_{PHH}}$				
50ER	Glucose	111.12	10.42	121.54
	Xylose	6.89	32.20	39.09
50WS	Glucose	112.70	10.86	123.56
	Xylose	8.77	33.38	42.14
50OP	Glucose	109.59	12.25	121.84
	Xylose	9.34	32.49	41.83

SS(H)F – Separate Hydrolysis and (co-)Fermentation
SS(C)F – Simultaneous Saccharification and (co-)Fermentation
AHH - hemicellulosic hydrolysate after autohydrolysis and concentration/detoxification
PHH - hemicellulosic hydrolysate after post-hydrolysis and concentration/detoxification

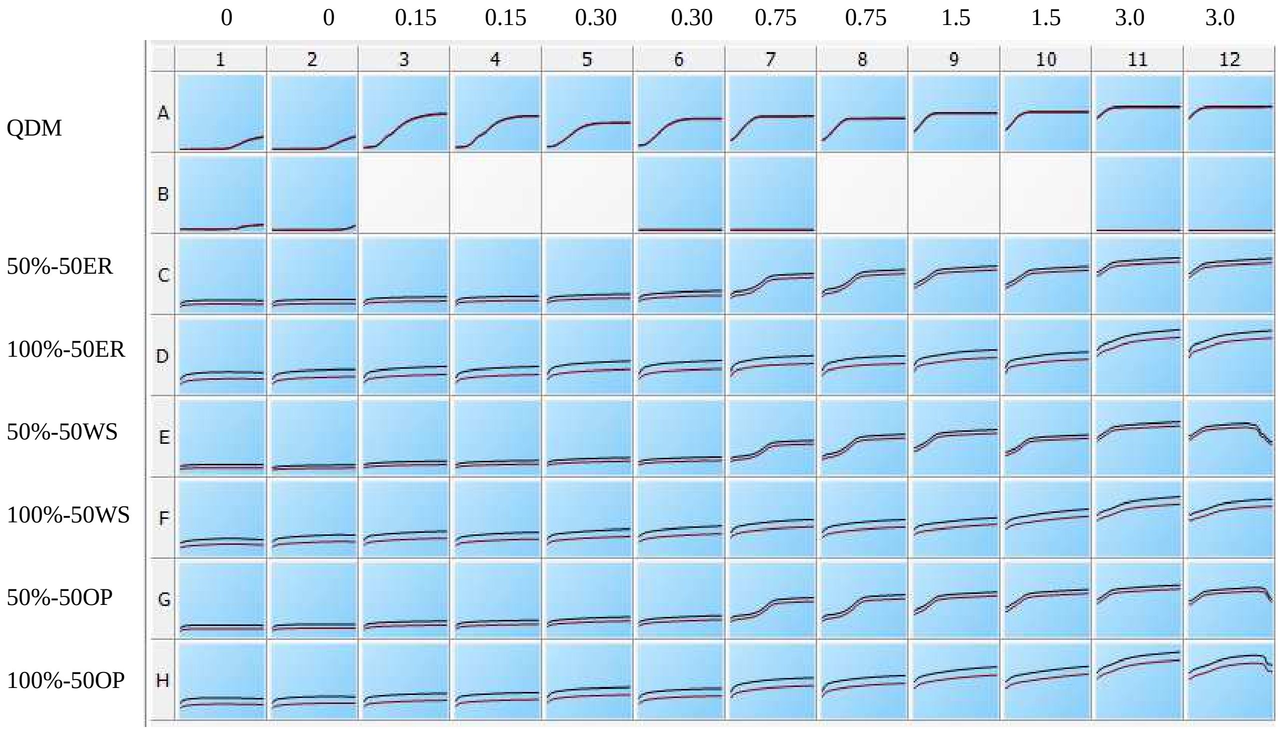
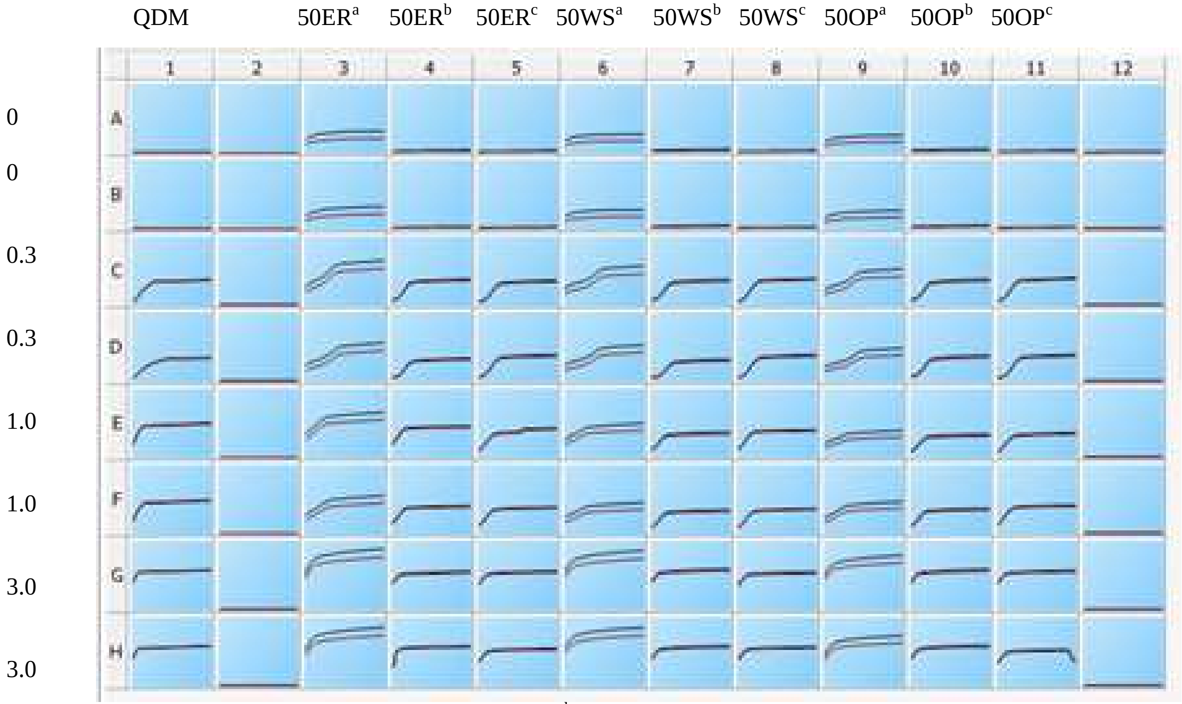


Figure 4.6 Microplate growth tests of *Saccharomyces cerevisiae* BH42 in (non-detoxified) hemicellulosic hydrolysate recovered after autohydrolysis at 220ºC from the 50ER, 50WS and 50OP. Growth was followed at 30ºC, 800 rpm, for 36 h, in 50% or 100% hemicellulosic hydrolysates using 0, 0.15, 0.30, 0.75, 1.5 and 3.0 g(CDW)/L yeast loading.



[a]hemicellulosic hydrolysate recovered after autohydrolysis and concentration (dilute 2-fold); [b]hemicellulosic hydrolysate recovered after autohydrolysis, concentration and detoxification (dilute 2-fold); [c]hemicellulosic hydrolysate recovered after post-hydrolysis, concentration and detoxification (dilute 2-fold).

Figure 4.7 Microplate growth tests of *Saccharomyces cerevisiae* BH42 in hemicellulosic hydrolysate recovered after autohydrolysis at 220ºC, or subsequent post-hydrolysis, from the 50ER, 50WS and 50OP. Growth was followed at 30ºC, 800 rpm, for 48 h, in 50% or 100% hemicellulosic hydrolysates using 0, 0.30, 1.0 and 3.0 g(CDW)/L yeast loading.

CHAPTER

5

FINAL REMARKS AND PERSPECTIVES

5.1 Final remarks

The seasonality and quantitative availability of a single lignocellulosic material is one the major barriers for the deployment of the 2G bioethanol technology and for the continuous and steady operation of biorefineries, especially in Southern Europe. In fact, the deployment of this technology is facilitated in the USA and Brazil, based on the large availability of lignocellulosic residues from the 1G bioethanol technology, like corn stover and sugarcane bagasse, respectively. In order to meet the requirement of biomass supply for the successful implementation of a commercial scale bioethanol biorefinery in Southern Europe, the use of feedstock mixtures was proposed in this work as an alternative to the use of individual feedstock. Three relatively abundant lignocellulosic biomasses available in this region were chosen: eucalyptus residues, wheat straw and olive tree pruning. These materials, used as model for 2G bioethanol production from feedstock mixtures, have different seasonalities, anatomies and chemical composition, which make this study of great interest. It fact, it embraces the processing of one residue from a cereal crop, and two distinct hardwoods. The choice of three combinations of ER, WS and OP as (ER:WS:OP); 50:25:25; 25:50:25 and 25:25:50, also represented as 50ER, 50WS and 50OP, was proposed according to relative complementary seasonality, i.e. according to their highest availability along the year, once ER is available through all the year, WS in late spring and early summer and OP mainly in winter and early spring. This concept of processing one agricultural and two hardwoods residues in different mixtures towards 2G bioethanol production is an innovative approach, to the best of my knowledge. This thesis proposes the integration of hydrothermal pretreatment with enzymatic hydrolysis and fermentation under different configurations. The co-fermentation of glucose (and other hexoses in minor amounts) and xylose present in hydrolysates to be fermented to bioethanol by a recombinant xylose-fermenting *Saccharomyces cerevisiae* strain.

In Chapter 2 (Part A), the study of the pretreatment of each individual feedstock allowed the understanding of the specific characteristics of each one and their impact in the different mixtures composition and processing. Under the conditions tested, extensive fractionation of cellulosic and non-cellulosic saccharides was achieved and the recovery of monosaccharides from each fraction was high. Despite the differences between the three feedstock, mainly in chemical composition, the same hydrothermal pretreatment (autohydrolysis) conditions could be applied to ER, WS and OP for maximal non-cellulosic saccharides recovery (liquid fraction), 210ºC (non-isothermal), and for maximal cellulose content (in solid fraction) and digestibility, 230ºC (non-isothermal). The monosaccharide-rich streams generated after the biorefining strategy proposed, using autohydrolysis and acid post-hydrolysis of the liquid fraction (non-cellulosic monosaccharides) and enzymatic hydrolysis of the pretreated solid fraction (cellulosic monosaccharide – glucose), have a composition apparently compatible to their use in biological or chemical conversion processes. This biorefining strategy was proposed, applied and developed in this thesis either for individual materials, using a cereal straw, two different hardwood residues, and their ternary mixtures, and can be probably applied to other individual lignocellulosic feedstocks and mixtures towards to provide sugar solutions for subsequent chemical or bioconversion processes. As the bioconversion processes will depend on the capacity of microbial cell factories to convert the different monosaccharides present in lignocellulosic hydrolysates and also to tolerate the inhibitors released or generated during biomass deconstruction (sugar degradation products, phenols, acids), different biorefining strategies to convert lignocellulose into fermentable sugars (monosaccharides) may be applied:

i) When a bioconversion process requires only glucose as carbon source and/or the microbial cell factory suffers from high inhibitory effect from biomass deconstruction products, the strategy involving autohydrolysis at 230ºC is preferential, using only the enzymatic hydrolysate of its solid fraction, i.e. glucose solution with low inhibitor content, as a simple SHF process. The enzymatic hydrolysis and bioconversion process can alternatively be performed simultaneously as SSF. This will be the case of using specific cell factories for the production of high-value chemicals (e.g. biosurfactants) and materials (e.g. PHB). The liquid fraction, containing sugar degradation products, can be directed to chemical processes for furans upgrading.

ii) When the viability of the bioconversion process is dependent of total lignocellulosic sugars, the conversion of both cellulosic and non-cellulosic sugar streams (from the 210ºC autohydrolysis process) should be performed with robust microbial cell factories able to convert hexoses and pentoses, as in the case of lignocellulosic ethanol production using industrial and recombinant pentose-fermenting *S. cerevisiae* strains. The enzymatic hydrolysis process can be performed before or simultaneously with the bioconversion step such as separate hydrolysis and (co-)fermentation (SH(C)F) or simultaneous saccharification and (co-)fermentation (SS(C)F). This has been the focus of Chapter 4.

iii) The use of mixed monosaccharides can be also applied for the production of other bio-based products as long as the microbial cell factories can tolerate the inhibitors present in the hydrolysate. The presence of glucose in non-cellulosic hydrolysates, like those resulting from WS and OP biorefining, can be beneficial to promote microbial growth and reduction of inhibitors in a first stage, and then to convert other sugar like xylose into value-added products like xylitol in a second stage. The major challenge will be to find cell factories specialized in the biosynthesis of value-added products and simultaneous robust enough to tolerate the in those hydrolysates.

The residual solids obtained after the enzymatic hydrolysis are rich in lignin, which might be suitable for lignin upgrading and commercialization or for use in co-generation to produce energy and steam (CHP) within the biorefinery.

Although the feasibility of the use of ER, WS and OP, in the saccharides recovery has been proved, the results obtained from the study of individual raw materials reinforce the interest and the requirement to study mixtures of lignocellulosic materials in biorefineries, because although the processing condition required for individual ER, WS and OP were in the same range (either for pretreatment or enzymatic hydrolysis), different compositions of the liquid and solid fractions recovered after pretreatment were obtained.

In Chapter 2 (Part B), the different lignocellulosic mixtures of 50ER, 50WS and 50OP generated very similar pretreated materials as denoted by the composition of both fractions, liquid and solid, although the content of each fraction slightly varied according to the contribution of the predominant feedstock. A range of pretreatment temperatures (205-220ºC) was identified as providing high saccharides recovery from the three different feedstock mixtures, but at 210ºC a maximal was found, which coincides with the optimal temperature for maximal saccharide recovery from individual feedstocks. Both factions recovered after autohydrolysis, liquid and solid, can be applied in several bioconversion processes with the advantage of having more similar composition than the simple use of different materials. These results are very relevant for the flexibility of biorefineries based on biochemical conversion processes, with special interest for Southern European biorefineries.

As the viability of using different feedstock mixtures in lignocellulosic biorefineries depends on further testing at the level of enzymatic hydrolysis of pretreated solids and bioconversion of both the cellulosic and hemicellulosic hydrolysates the study of these processes was performed in the last two Chapters of this thesis. The enzymatic hydrolysis of the solid fraction recovered after autohydrolysis of individual feedstock (ER, WS and OP) was studied using a commercial enzymatic formulation (Celluclast® 1.5L and Novozyme® 188). The statistical analysis of the variables studied, namely pretreatment temperature, enzyme dosage and solid loading, revealed that all had an impact on glucose concentration and yield. Despite the pretreatment temperature had a great impact in glucose yield, especially for 50ER, the pretreatment temperature conferring higher cellulose digestibility was above 220ºC for all individual feedstocks and their mixtures. Anyway, maximal cellulose digestibility was found at different pretreatment temperatures for each feedstock and their mixtures (see below) (Chapter 3), which contrasts with the observed for fractionation of non-cellulosic sugars and their maximal recovery in the liquid fraction, where the same pretreatment temperature (210ºC) was found to be optimal for the three feedstocks and their mixtures (Chapter 2). Also, a relatively high enzyme dosage was required for high glucose recovery although this may have a negative impact on the economy of the process due to the still high enzyme cost. Finally, the solid loading had the most obvious impact on glucose concentration, with higher concentration (>100 g/L) but lower yield of glucose when enzymatic hydrolysis was performed at 20% (w/v) solids and lower concentration and higher yield (>95%) of glucose when it was performed at 5% (w/v). Water and energy economy requires the operation of biorefineries at high solid content and thus the process was optimized for the higher solid content tested (20% w/v). Two different approaches were used to optimize enzymatic hydrolysis of feedstock mixtures at high solid loading (20% w/v): i) a single condition was defined for all feedstock mixtures, equivalent to an equal proportion of each feedstock: autohydrolysis at 224ºC and enzyme loading of 0.63 g/g_{solids} and ii) three conditions were defined according to the predominant feedstock in each mixture: autohydrolysis at 222, 230 or 228ºC and enzyme loading of 0.75, 0.54 and 0.75 g/g_{solids}, for 50ER, 50WS and 50OP, respectively. At the end similar glucose concentration and yield were achieved, corresponding to more than 85 g/L and 80%, respectively, for all the feedstock mixtures. The results obtained with the two different approaches, revealed high glucose recovery and flexibility on the choice of the enzymatic hydrolysis conditions for processing feedstock mixtures. However, the feasibility of the process involving mixtures of lignocellulosic biomasses will be depended of a cost analysis and performance trade-off between the enzyme dosage (and associated cost) and the energy consumption for pretreatment. For the complete feasibility study, the use of the liquid fraction recovered after pretreatment should be taken into account since this fraction still contains a significant amount of sugars and the use of high pretreatment temperatures generates larger amounts of sugar degradation products.

Finally, in Chapter 4 the conversion of glucose and xylose from mixtures 50ER, 50WS and 50OP into bioethanol was carried out, by integrating pretreatment, enzymatic hydrolysis and fermentation steps under different process configurations. For this purpose, a recombinant xylose-fermenting *S. cerevisiae* strain (BH42) was used in the fermentative process. The pretreatment temperature used to demonstrate the feasibility of converting mixtures of lignocellulosic materials into bioethanol was 220ºC. The models obtained in enzymatic hydrolysis optimization (Chapter 3) were useful to predict the enzymatic dosage for the pretreatment temperature at 220ºC. The liquid fraction recovered after autohydrolysis and post-hydrolysis (when applied) was concentrated, allowing the increase of saccharides and decrease of volatile toxic compound (e.g. furfural). Although the detoxification was performed to decrease the concentration of the potential inhibitors of fermentation, a high concentration of organic acid was still observed. As consequence of the inefficient detoxification process, in SSCF an accumulation of glucose in the medium was observed, mainly in the mixture containing highest OP proportion (50OP) and only in the experiments performed with higher xylose concentration (post-hydrolysates) a more expressive consumption of this pentose was observed. In SHCF, no glucose accumulation was observed, and the performance using the different feedstock mixtures was very similar and higher than in SSCF, however with lower xylose consumption. Accordingly, both SSCF and SHCF processes can be applied generating similar ethanol yields and reaching ethanol concentrations higher than 40 g/L.

Some important scientific contributions, beyond the state-of-the-art, have been provided with this thesis:

- The composition of pretreated feedstock mixtures, both liquid and solid fractions, corresponded to the proportional composition of pretreated individual feedstocks processed under the same conditions. This observation suggests that the composition of other pretreated feedstock mixtures can be predicted by using the composition of individually pretreated feedstock in the respective proportions.
- The approach used for the optimization of enzymatic hydrolysis of pretreated solids, i.e. the development of models with the results obtained with the enzymatic hydrolysis of individual feedstock to be used for prediction of enzymatic hydrolysis of pretreated feedstock mixtures, can be used for future works involving other mixtures.
- The production of more than 40 g/ L ethanol from 20% (w/v) of pretreated solids of different mixtures containing ER, WS and OP, demonstrate the feasibility of converting mixtures of

lignocellulosic materials, including those of very different nature like straw from cereal crops and hardwood residues, into 2G bioethanol.

- This work further provides the basis and hints for further process development and scaling up for the production of bioethanol from lignocellulosic mixtures containing agricultural and hardwood residues.

This thesis is expected to contribute to the deployment of multi-feedstock 2G bioethanol biorefineries in Portugal, in Europe and worldwide.

5.2 Perspectives

Future experimental work should be less focused on biomass pretreatments, exhaustively covered in this thesis, and more on the biochemical conversion of hydrothermal pretreated feedstocks (individual and in mixtures), but also on smart process intensification.

Among the most promising approaches to improve the conversion of mixtures containing lignocellulosic materials like ER, WS and OP are:

- The processing of feedstock mixtures by hydrothermal pretreatment at higher solid lading (e.g. 40 g/60 g water, instead of approx. 14 g/100 g water), contributing to process intensification, by increasing the concentration of sugars in the hemicellulosic hydrolysates and by allowing the direct bioconversion of the whole slurry (solid and liquid fraction) without separation;
- The use of last generation commercial enzyme preparation (e.g. Cellic CTec3), towards minimal enzyme loading and minimal enzyme cost;
- The use or development of more robust and efficient recombinant xylose-fermenting *S. cerevisiae* strains, or even more advanced strains able to directly convert oligo- or polysaccharides by the expression of hydrolytic enzymes – Consolidated Bioprocessing (CBP).

However, the results obtained give several hints to further integrate and optimize processes to efficiently convert feedstock mixtures containing ER, WS and OP into 2G bioethanol:

- The effect of hemicellulosic hydrolysates and their individual components in yeast performance, *S. cerevisiae* BH42 or other xylose-fermenting strains, is of particular interest. The furans, the organic acids and the phenolic compounds are described as the molecules showing higher antimicrobial activity. A especial focus should be given to the phenolic compounds from OP, as denoted by the higher inhibitory effect observed with 50OP.
- The inhibitory effects of hemicellulosic hydrolysates can be mitigated by higher yeast loading and/or better adaptations protocols, namely those applying progressive adaptation through fed-batch approaches. More advanced approaches will be the selection of improved strains through long-term adaptation or evolutionary engineering, for optimal performance in specific substrates, including feedstock mixtures.
- Although SSCF and SHCF of pretreated feedstock mixtures achieved relatively high ethanol concentration, those were performed in shake flasks, and the scaling up for a bench bioreactor, with the possibility of operating under fully controlled conditions (e.g. pH and available oxygen) will certainly be crucial to improve the bioconversion process.

Although the technical feasibility of converting mixtures of ER, WS and OP into ethanol was demonstrated in this thesis, it is necessary to evaluate the sustainability of the value-chain, performing a life cycle assessment (LCA) mixtures of to evaluate the mass and energy inputs and outputs, and potential economic and environmental impacts from “cradle to gate”. Of particular interest will be the study of the benefits of operating a multi-feedstock biorefinery *versus* a single-feedstock biorefinery in Southern Europe.