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LIGNOCELLULOSIC RESIDUES VALORIZATION USING A COMBINED APPROACH OF SUB-, SUPERCRITICAL FLUIDS AND BIOTECHNOLOGY

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Ao meu marido e ao meu filho, a minha VIDA

“Se, no princípio, a ideia não é absurda, então não há esperança para ela.”

Albert Einstein

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(Aqueles que me conhecem bem, sabem que os agradecimentos não vão ser pequeninos... Mas tem que ser. Ensinaram-me que os melhores trabalhos só se fazem com boas equipas e, este, não foi exceção. Embora apenas com o meu nome, foi toda esta equipa que o desenvolveu sem a qual nunca teria sido capaz de apresentar este trabalho.)

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Resumo

O trabalho apresentado nesta tese propõe o desenvolvimento de um processo integrado que combine fluidos sub-, supercríticos e biotecnologia de forma a criar uma cadeia de valorização para resíduos lenhocelulósicos. A água sub-crítica é avaliada como um meio alternativo e viável para proceder, simultaneamente, a reacções de extracção de compostos de alto valor acrescentado directamente disponíveis nos resíduos lenhocelulósicos como, por exemplo, compostos fenólicos assim como para reacções de hidrólise dos principais componentes estruturais, celulose, hemicelulose e lenhina. Foram estudados diferentes resíduos lenhocelulósicos tais como o resíduo de papel reciclado, as massas vínicas e o bagaço de maçã. Relativamente à reacção de hidrólise, o principal objectivo foi a obtenção de soluções aquosas ricas em açúcares que poderão, posteriormente, ser utilizadas como fonte alternativa de carbono para o crescimento de leveduras oleaginousas que são, por sua vez, capazes de acumular lípidos e carotenóides. Como estágio final do processo proposto, uma fonte alternativa de óleo foi aplicada para a produção de biodiesel através de um processo integrado e em contínuo que combina as tecnologias de dióxido de carbono supercrítico e enzimas. Desta forma, a cadeia de valorização proposta será capaz de gerar, a partir de resíduos lenhocelulósicos, três compostos de alto valor acrescentado: lípidos, carotenóides e biodiesel, combinando pela primeira vez, vários solventes alternativos, bioengenharia e biotecnologia.

O residuo de papel reciclado foi utilizado como um residuo lenhocelulósico modelo para avaliar o potencial da água-subcrítica na hidrólise da celulose, hemicelulose e lenhina. Foram estudados dois modos de operação distintos: batch e semi-contínuo. Após uma detalhada análise de todo o processo incluindo a avaliação de diferentes parâmetros operacionais, a recuperação dos produtos finais e o desenvolvimento de balanços de massa detalhados, foi possível provar que o modelo em semi-contínuo provou ser o desenho de processo mais adequado com uma maior recuperação de compostos solúveis, maioritariamente monossacáridos, evitando ao mesmo tempo a formação de compostos de degradação.

As massas vinicas foram utilizadas para avaliar o potencial de utilização de um tratamento com água-subcrítica onde simultaneamente se extrai compostos fenólicos disponíveis ao mesmo tempo que se hidroliza os seus principais componentes de forma a obter uma solução aquosa rica em açúcares. A água-subcritica provou ter o potencial para ser usada tanto como solvente para extracção de compostos de alto valor acrescentado assim como reagente para reacções de hidrólise.
A extracção de antioxidantes utilizando água-subcrítica apresentou rendimentos 10 vezes superiores aosobtidos usando solventes convencionais. Adicionalmente, a água-subcrítica permitiu uma rápida recuperação de mais de 60% de carbohidratos estruturais das massas vínicas, maioritariamente, na forma de oligossacáridos que podem ser, posteriormente, digeridos por enzimas ou utilizados

directamente por microorganismos como a levedura *Rhodotorula babjevae*. Esta levedura atingiu DO's 2 a 5 vezes maiores que aquelas obtidas durante a utilização de misturas de açúcares modelo demonstrando assim, pela primeira vez, que os resíduos agrícolas quando tratados com água-subcritica, originam uma fonte de carbono adequada para o crescimento de leveduras.

Foram testadas cinco leveduras oleaginosas para demonstrar o potencial da utilização do bagaço de maçã como fonte de carbono não só para efectuar o crescimento das leveduras mas também para a produção de lípidos e carotenóides. Foram observados resultados muito positivos tanto para o crescimento como para a acumulação de lípidos e carotenóides sem efectuar qualquer optimização dos parâmetros de crescimento das leveduras.

Foi utilizada uma fonte de óleo alternativa, o óleo usado, como matéria-prima para a produção de um combustível alternativo, o biodiesel, através da sua aplicação num processo integrado em contínuo utilizando dióxido de carbono supercrítico e um reactor de leito fixo enzimático. A prova de conceito foi efectuada através da recuperação de um produto final com uma pureza superior a 96 % em metil estéres de ácidos gordos através da utilização de um processo baseado na tecnologia de dióxido de carbono supercrítico, seja o material de partida um óleo virgem ou um óleo usado.

Palavras-Chave: Residuos lenhocelulósicos, água-subcrítica, carbohidratos, leveduras oleaginosas, fluidos supercríticos, compostos de alto valor acrescentado

Abstract

An integrated processed combined sub-, supercritical fluids and biotechnology was proposed to create a valorization chain for the lignocellulosic residues. Hot compressed water was evaluated as an alternative medium to perform the simultaneous extraction of added value compounds directly available in the lignocellulosic residues such as phenolic compounds as well as the hydrolysis of the main structural components, cellulose, hemicellulose and lignin. Different lignocellulosic residues such as recycled paper mill sludge, grape pomace and apple pomace were studied. The main goal of the hydrolysis was to obtained aqueous solutions rich in sugars which can be further used as alternative carbon source to oleaginous yeasts growth which are capable of accumulating lipids and carotenoids. As final stage of this proposed process, the use of alternative oil sources was evaluated by using an integrated continuous process, which combined supercritical carbon dioxide and enzymes. Therefore, this valorization chain will be able to generate form lignocellulosic residue, three added value compounds: lipids, carotenoids and biodiesel by combining for the first time several alternative solvents, bioengineering and biotechnology.

Recycled paper mill sludge (RPS) was used as lignocellulosic residue model to evaluate the potential of using HCW to perform the hydrolysis of the three main components, cellulose, hemicellulose and lignin. Two different operational modes were tested: batch and semi-continuous flow reactors. From a detailed process analysis including the evaluation of different operational parameters, target products recovery and the devolvement of detailed mass balances, the semi-continuous model proved to be the most fitted process design, with a higher recovery of water soluble compounds, mainly monosaccharides, avoiding at the same time the formation of degradation compounds.

Grape pomace was used to evaluate the potential of using a HCW treatment, which simultaneous extracts the phenolic compounds directly available in the residue as well as the hydrolysis of its main structures in order to obtain a solution rich in sugars. HCW proved to have the potential to be used as a solvent for the extraction of added-value compounds as well as a reactant for the hydrolysis. The extraction of antioxidants using HCW presented yields which are 10 times higher than those using conventional solvents. Additionally, HCW hydrolysis allows fast recovery of up to 60% of structural carbohydrates of grape pomace mainly in the form of oligosaccharides, which can be further digested by enzymes or used directly by microorganisms such as *Rhodotorula babjevae* yeast. This yeast exhibited an OD 2 to 5 fold higher than obtained with model mixtures, demonstrating, for the first time, that HCW-treated agricultural residues affords suitable carbon source for the growth of non-Saccharomyces yeast.

Five different oleaginous yeasts strains were tested to demonstrate the suitability of apple pomace hydrolysates material as carbon source not only for growth but also for lipids and carotenoids production. Good performances in terms of growth, lipids and carotenoids accumulation were observed without performing any growth operational conditions optimization.

Alternative oil sources, such as waste cooking oil, was used as raw material to produce an alternative biofuel- biodiesel applying a combined integrated continuous process using SC-CO2 and a continuous enzymatic flow reactor. It was made the proof of concept by achieving a product with >96 % of FAME using a $ScCO_2$-based process as described, whether the starting material is virgin or waste cooking sunflower oil.

Key words: Lignocellulosic residues, hot compressed water, carbohydrates, oleaginous yeasts, supercritical fluids, added value compounds

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Abbreviations, symbols and constants

5- HMF – 5- Hydroxymethylfurfural

AP - Apple Pomace

CO_2 – Carbon dioxide

DP – Degree of polymerization

DW – Dried weight

et al.- *et alli;* and others

FAME - Fatty-Acids Methyl Esters

FFA - Free Fatty Acids

Fru – Frutose

g – gram

GAE – Gallic acid equivalent

Glu - Glucose

GC - Gas Chromatography

GP - Grape pomace

HCW – Hot compressed water

i.e. – id est; in other words

K - degree Kelvin

LBET - Lobry de Bruyn-Alberda van Ekenstein transformation

LB – Lipid body

min - minute

mM – millimolar

P - pressure

Pc - critical pressure

ppm - parts per million

RPS – Reclycled Paper Mill Sludge

R^2 - coefficient of determination

Ro – severity factor

s - second

$scCO_2$ - supercritical carbon dioxide

SCF - supercritical fluid

T- temperature

t – time

T_b - base temperature

Tc – critical temperature

T_i - operational temperature

TAG – triacylglycerols

UV/Vis - Ultraviolet/Visible

Xyl - Xylose

YNB- Yeast Nitrogen Base

YMA- Yeast malt agar

ºC - degree Celsius

wt% - weight percentage

1

Introduction

1.1. Biorefinary concept- The role of Bioengineering Systems

Sustainability can be seen as a dynamic process with the final goal of achieving a basic supply for world's population needs and stabilization by reducing hunger and poverty while adequate living standards are maintained while preserving Earth's ecosystem (Kates and Parris, 2003). Most predictions indicate that oil production peak will be reached in the early twenty-first century resulting in a decrease of oil production at the same time world oil demand will continue to increase. According to the U.S. Energy Information Administration (EIA), emerging economies outside the OECD (Organization for Economic Cooperation and Development) will increase world energy consumption ca. 50% between 2009 and 2035 accounting for 84% of growth in world energy use. Developed OECD nations are expected to account for only 14% (Virmond et al., 2013). Additionally to the oil crisis, according with *Meadows et al.* with the report "The Limits of Growth" to current days, food consumption will reach a maximum at 2020 along with an increase of the world population to 8600 million people in 2040 at the same time that a sharp decrease of natural sources availability will occur (Arai et al., 2009). These projections will have a strong impact on world's sustainability forcing industries to seek sustainable and alternative sources for energy and food production (Clarck, J.H;Deswarte, F.E.I.;Farmer, 2009).

During the last recent years, the world has been taking substantial steps into the transition to a biobased economy. The decrease in the availability of fossil fuels, serious problems arising from climate change, policy and economical world crises are steering the world economy into a scenario where material wastes are minimized, new bioproducts are replacing their fossil equivalents, greenhouse gas (GHG) emissions are reduced forcing the society to acquire environmental friendly habits and new live styles. The use of biomass for fuels production could represent a carbon-neutral or near to carbon-neutral lifecycle if the non-renewable inputs during biomass treatment such us fertilizers consumption, harvest and transportation of large amounts to distant locations would be reduced or eliminated (Wu et al., 2005). An economy based on innovative and cost-efficient use of biomass for the production of biobased products and bioenergy should be driven by well-developed, integrated biorefinary systems (Jong and Jungmeier, 2015). According with EIA, biorefining is "the sustainable processing of biomass into a spectrum of marketable products and energy" (Ruiz et al., 2013). Three biorefinary systems are currently being developed: (1) the "Whole Crop Biorefinary", using cereals or maize as raw material, (2) "Green Biorefinary", using "nature-wet" biomasses, such as green grass or immature cereal and (3) the "Lignocellulose Feedstock Biorefinary", using "nature-dry" raw material, such as cellulose-containing biomass and wastes (Biorefinary – Systems).

It is estimated a biomass utilization increase to 243-316 million tones by 2030 (Möller et al., 2011) with a corresponding potential supply of about 50% of the world primary energy consumption by the year of 2050 (Wu et al., 2008). In figure 1.1, it can be observed a flowchart model of the significant

variety of biobased products that can be obtained by biomass feedstock's (Kamm, B.; Gruber, P.R.; Kamm, 2008).

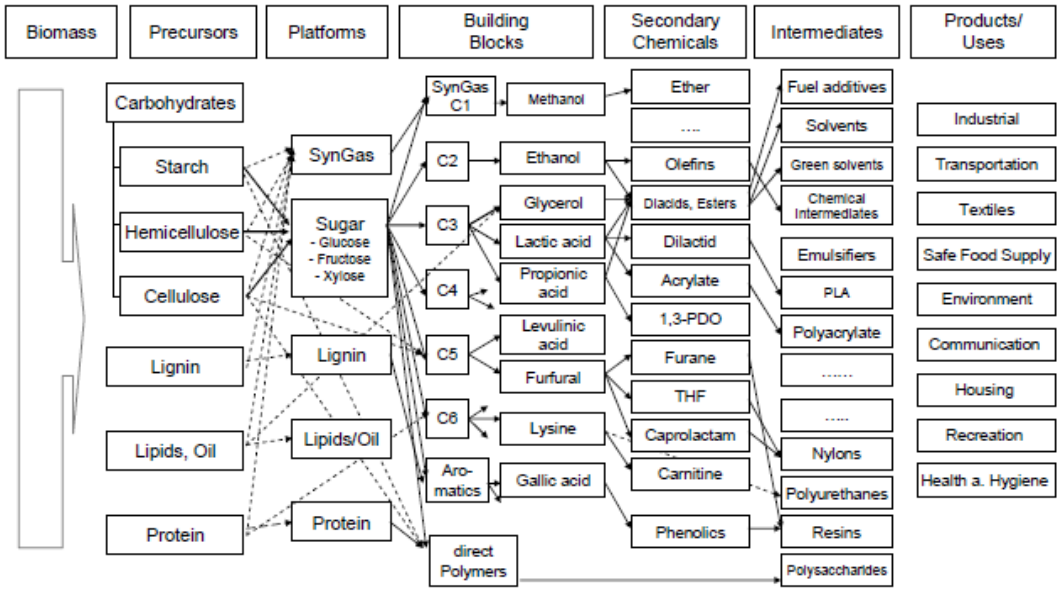


Figure 1.1: Flowchart model of biobased products from biomass feedstock's (Kamm, B.; Gruber, P.R.; Kamm, 2008)

It can be observed from figure 1.1 several possible applications for sugars derived from lignocellulosic materials depending on their carbon chain length such as ethanol for transportation or lactic acid for food supply. These building blocks can also be further transformed into secondary chemicals belonging to several categories such as fuel additives, resins, etc. It is very important to evaluate the strengths and weaknesses of all the bioengineering systems involved in biorefinaries to enable the integration of different technologies and feedstocks, maximizing the diversity of applications and products formed (Budarin et al., 2011). Biomass chemical industry needs to be based on a local production model against a centralized mass production applied in the petrochemical industry. The optimum production process scale should be determined considering the minimum energy consumption per life cycle of products. Biomass transportation energy will be much higher than in petrochemical industry due to its highly dispersed nature and relatively low energy density (Arai et al., 2009). A biorefinary concept is based in the application of the Green Chemistry principles mainly the use of sustainable feedstocks, the use of safer processes, energy demands should be minimized not only during the production as well as during the product life cycle, hazardous chemical uses and production should be avoided, and the final product should be non-toxic, environmentally friendly and easily recyclable with minimum production of waste (Clarck, J.H;Deswarte, F.E.I.;Farmer, 2009).

1.2. Lignocellulosic materials: Structure and characterization

Plant cell walls are mostly composed by carbohydrate polymers (cellulose, hemicellulose) and an aromatic polymer (lignin). These polymers represent the major constituents of the cell walls in plant cells and are organized into a lignocellulosic structure represented in figure 1.2.

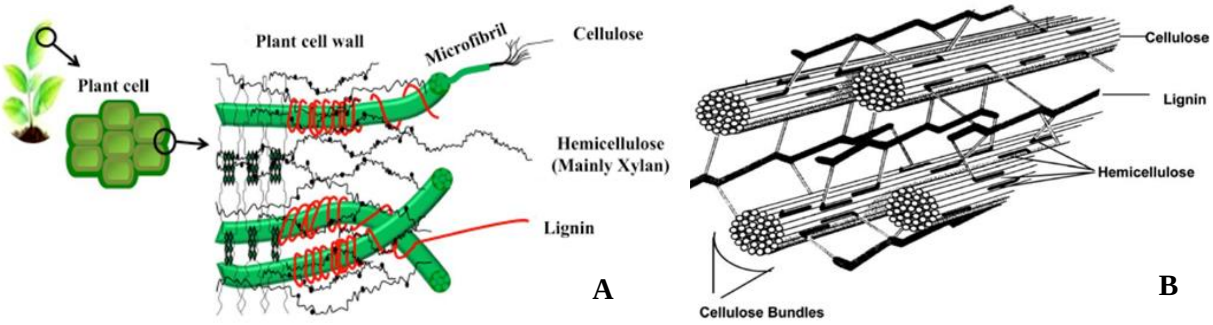


Figure 1.2: Lignocellulosic biomass: (A) Cellulose, hemicellulose and lignin as major constituents of cell wall (Tomme, P.; Warren, R. A. J.; Gilkes, 1995); (B) Lignocellulosic structure (Wu et al., 2008).

In addition to the main components, natural lignocellulosic materials contain also a small amount of pectin, nitrogenous compounds and minerals. For instance, the element content of wood is about 50 % carbon, 6 % hydrogen, 44 % oxygen, and 0.05–0.4 % nitrogen (Chen, 2014). Detailed information about each one of the main components is described in the next section.

1.2.1. Cellulose

Cellulose has an annual estimated production of 50 billion tones being the most abundant polymer on earth (Wu et al., 2008). It is consisted by carbon (44.44 %), hydrogen (6.17 %) and oxygen (49.39 %). The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ with n is called the degree of polymerization (DP), determined by the number of monomers that compose each cellulose chain ranging from hundreds to thousands or even higher (Chen, 2014).

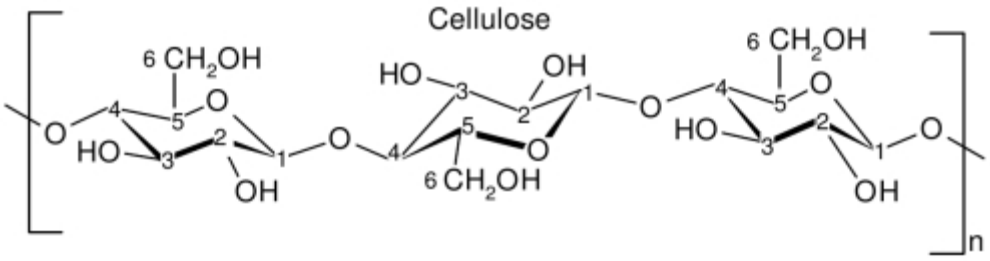


Figure 1.3: Cellulose molecular structure (Mohan et al., 2006).

As the main component of lignocellulosic wastes, cellulose is a skeletal polysaccharide consisting of a linear chain of several D-glucose units linked by β-1,4- glycoside linkages (Figure 1.3)(Mohan et al., 2006). Glucose chains, also called elemental fibrils are set in parallel and aligned side-by-side in a specific crystalline arrangement forming a beam called the microfibrils. Microfibrils are, in turn, bundled together to form cellulose fibers through covalent bonds, hydrogen bonds and Van der Waals forces (Tomme, P.; Warren, R. A. J.; Gilkes, 1995). Microfibrils are thus composed of elementary fibrils that are further associated with noncellulosic polymers. Each cellulose microfibril has approximately 36 glucose chains (Festucci-Buselli et al., 2007). The degree of polymerization of cellulose chains is around 2,000-25,000 glucose residues (Delmer, 1999) and the fibril contains approximately 60–80 cellulose molecules (Chen, 2014).

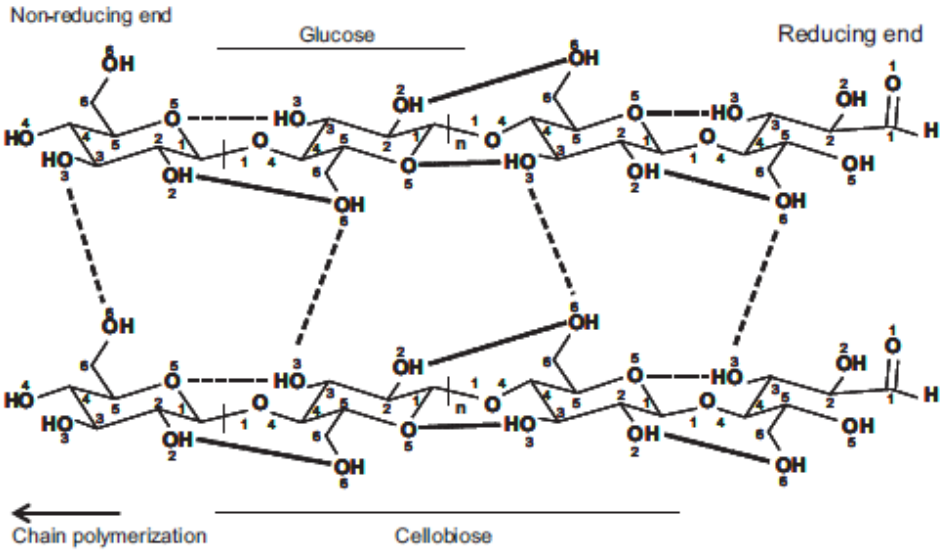


Figure 1.4: Cellulose structure and inter- and intra-chain hydrogen bonding pattern. Dashed lines: inter-chain hydrogen bonding; Dotted lines: intra-chain hydrogen bonding (Structure, organization, and functions of cellulose synthase complexes in higher plants.)

The basic repeating unit of cellulose is cellobiose, the β-(1-4)-linked disaccharide of D-glucopyranose. The multiple hydroxyl groups on the glucose present in one chain of the cellulose structure form hydrogen bonds with oxygen atoms on the same or with a neighbor chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength. Two different ending groups are found in each cellulose chain edge. A non-reducing group is present where a closed ring structure is found and a reducing group with both an aliphatic structure and a carbonyl group is found at the other end of the chains (Figure 1.4) (Festucci-Buselli et al., 2007).

Cellulose can be considered as a condensation polymer of glucose with an average molecular weight of 300.000-500.000 glucose monomer units depending on DP which varies from 7000-10.000 for

wood to as high as 15.000 for cotton (Bobleter, 1994). The rigid structure and extensive hydrogen bond network are responsible for its crystalline structure causing a low-surface area for contact. This makes cellulose hard to be degraded and insoluble in water as well as most organic solvents. Cellulose can be found in different degrees of crystallinity from highly ordered crystalline regions to amorphous ones or with lower order. The degree of crystallinity also varies according with cellulose nature and pre-treatment. For instance, cotton is 70% crystalline and more ordered than wood while regenerated cellulose can achieve a crystallinity of 40% (Wu et al., 2008). Cellulose crystallinity of 90 -100% are found in plant-based fibres such as corn stover or wheat straw and 60–70% in wood based fibres were determined. (Thygesen et al., 2005) . It was reported a cellulose degree of crystallinity in grape skin of 66.1.% for the Touriga Nacional variety (Mendes et al., 2013) whereas for pine wood, depending on weather exposure and the applications of coatings, de degree of crystallinity of cellulose can vary from 22.1 to 63.1% (Lionetto et al., 2012).

Cellulose reactivity depends on its physical and chemical structure. Indeed, crystalline regions tend to react slowly and are chemically more resistant than amorphous regions (Delmer, 1999).

1.2.2.Hemicellulose

Hemicellulose is another main component in plant fiber materials. In association with cellulose in cell wall, hemicellulose is a branched amorphous polysaccharide consisted of several polymerized pentoses (mainly xylose and arabinose) and hexoses (galactose, glucose and mannose) as well as 4-O-methyl glucuronic acid and galacturonic acid residues linked together by β-1,4- and occasionally β-1,3- glycosidic cobonds (Figure 1.5)(Chen, 2014; Mohan et al., 2006).

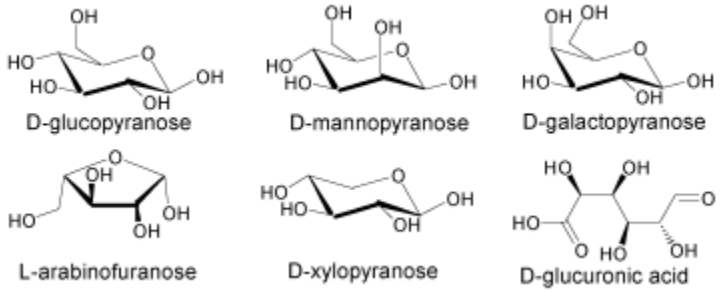


Figure 1.5: Main constituents of hemicelluloses (Hansen and Plackett, 2008).

The content and structure of hemicellulose varies depending on the plant material. Comparing with cellulose, hemicellulose has amorphous structure and branches with short lateral chains varying from less than 100 to about 200 units (Goldstein, 1981) of different sugars. Hemicellulose bounds to the cellulose microfibrils via hydrogen bound and covalently attaches to lignin to form the highly complex structure of plant cell wall (Yang, 2015) Hemicelluloses have highly heterogeneous

noncrystalline structures, therefore it can be easily hydrolyzed by dilute acid, base and myriad hemicellulose enzymes to monomeric sugars and other valuable chemicals (Dutta et al., 2012).

It is generally believed that hemicelluloses main components are *xyloglycans (xylans)*, *mannoglycans (mannans)*, *xyloglucans and arabinogalactans* (Ebringerová, A.; Hromádková, Z.; Heinze, 2005). These main components present structural variations differing in side-chain types, polysaccharides distribution, localization and/or glycoside linkages distribution in the macromolecular backbone (Hansen and Plackett, 2008). Further detailed information about each one of the main structures of hemicellulose will be presented in the next sections.

Xylans

Xylan-type polysaccharides are the main hemicellulose components constituting about 20–30% of hardwoods biomass and herbaceous plants. In some plant materials such as grasses and cereals xylans can occur up to 50% (Ebringerová and Heinze, 2000). Therefore, xylans are highly available in vast amounts as by-products from forestry, food, agriculture, wood, and pulp and paper industries (Ebringerova et al., 2005). Among hemicelluloses, xylan was the most abundant component in *Vitis vinifera L.* red grape pomace stalks (ca. 12%) (Prozil et al., 2012).

In most cases, xylans are heteropolymers with a homopolymeric backbone chain of 1.4-linked β-D-xylopyranose units with short carbohydrate side chains on the 2- or 3-position. These side chains comprise D-glucuronic acid or its 4-O-methyl ether, L-arabinose and/or various oligosaccharides, composed of D-xylose, L-arabinose, D- or L-galactose and D-glucose (Hansen and Plackett, 2008). Depending on xylans primary structure, these can be further divided into two main classes: **homoxylans** with a backbone consisted only by D-xylopyranose units linked by β- (1 → 3) or mixed β-(1 → 3, 1 → 4)-glycosidic linkages and **heteroxylans** that are polysaccharides with a backbone composed of 1,4-linked β-D-xylosyl residues where some of these xylosyl residues are substituted with 4-O-methyl α-D-glucuronic and occasionally with α-D-glucuronic acid at position 2, in case of hardwoods or, as in case of grasses, the xylan backbone is predominantly substituted with α-L-arabinofuranosyl residues in position 2 or 3. (Mazumder, K.; Peña, M.J; O'Neill, M.A; York, 2012). Heteroxylans with substituents of α-D-glucuronic acid or 4-O-methyl-α-D-glucopyranosyl uronic acid residues are called glucuronoxylans and heteroxylans with α –Larabinofuranose residues are called arabinoxylans. There are even more complex heteroxylans substitution patterns such as (arabino)glucuronoxylans, (glucurono)arabinoxylans (Ebringerová and Heinze, 2000). Arabinoxylan has been identified in a variety of the main commercial cereals such as wheat, rye, barley, oat, rice, and corn, sorghum as well as in other plants such as rye grass, bamboo shoots, among others (Ebringerova et al., 2005). Apple hemicellulose composition was analyzed indicating the presence of

complex heteroxylans such as fucogalactoxyloglucans and arabinoxylans as well as pectic arabinans and galactans, glucomannans, and 1,3 linked glucans (Voragen et al., 1986).

Softwood arabinoglucuronoxylan has a backbone of β-(1→4) linked xylopyranose units (Figure 1.6) with single-unit side chains of 4-O-methyl-D-glucuronic acid units attached by α-(1→2) bonds, on average one unit per 5-6 xylose units, and L-arabinose units attached by α-(1→3) bonds, on average one unit per 5-12 xylose units.

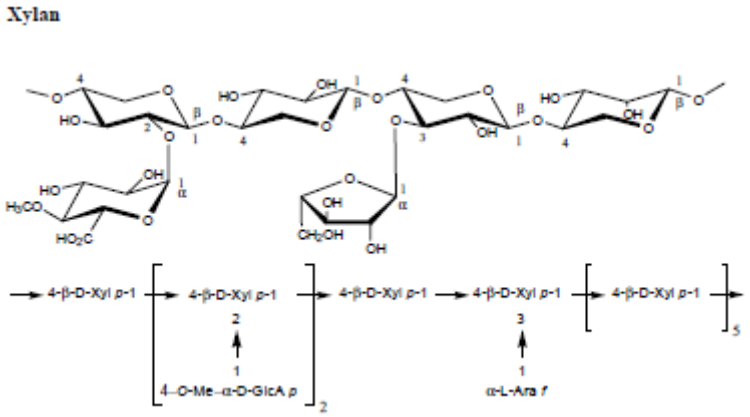


Figure 1.6: Structure of a heteroxylan hemicellulose - softwood arabinoglucuronoxylan (Shimizu, 2001).

Mannans

Mannans are the major constituents of the hemicellulose fraction in softwoods and show wide spread distribution in plant tissues (Moreira and Filho, 2008). It also represents a major part of hemicelluloses in grape pomace together with galactans (Mendes et al., 2013). The mannan-type polysaccharides of higher plants can be divided in four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomanan (De et al., 2001). Each of these polysaccharides present a β-1,4-linked backbone containing mannose or a combination of glucose and mannose residues. Additionally, the mannan backbone can be substituted with side chains of α-1,6-linked galactose residues (Liepman et al., 2007).

Linear mannans are homopolysaccharides composed of linear main chains of 1,4-linked β-D-mannopyranosyl residues and contain less than 5% of galactose and they are the major structural units in woods and in seeds of many plants, such as ivory nuts and green coffee beans (Aspinall, 1959). In galactomannans, the backbone is made up exclusively of β-(1→4)- linked D-mannopyranose residues in linear chains with side chains of single1,6-linked α-D-galactopyranosyl groups attached along the chain whereas in glucomannans, the backbone has both β-(1→ 4)-linked D-mannopyranose and β-(1→ 4)-linked D-glucopyranose residues in the main chain. Both types of mannoglycans have varying degrees of branching with D-galactopyranose residues in the 6-position of the mannose backbone.

Differences in the distribution of D-galactosyl units along the mannan structure are found in galactomannans from different sources (Moreira and Filho, 2008).

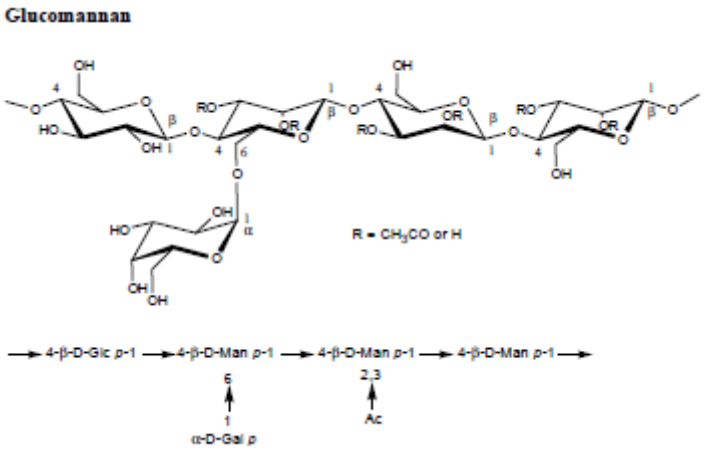


Figure 1.7: Glucomannan strucuture (Shimizu, 2001).

Galactoglucomannans are a more complex hemicellulose mannan-type consisting of a mannose backbone composed of β-(1-4) linked D-mannopyranosyl units combined with a lower number of β(1-4) linked D-glucopyranosyl units. Branched to the mannopyranosyl units, there are side groups of single (1-6)-linked α-galactopyranosyl units. In softwood, the **galactoglucomannans** are O-acetylated at C-2 and C-3 of the mannosyl units (Lundqvist et al., 2002).

Xyloglucans

Xyloglucan is known mainly as a polysaccharide in the primary cell wall of higher plants with the same backbone as cellulose with β-(1-4)-linked D-glucose units but with side chains attached at the hydroxyl group of C6. The side chains consist either of single xylose units or of galactose, arabinose or fucose units (12)-linked to xylose. In softwood, xyloglucan is only a minor compound (Sjostrom;E., 1993).

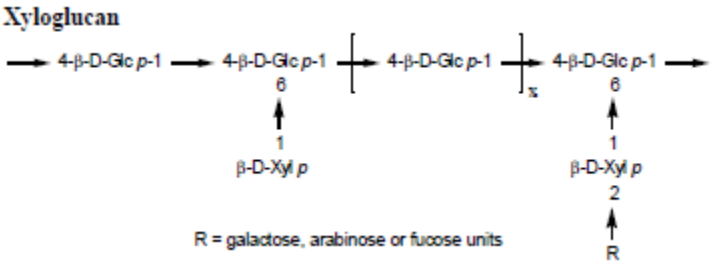


Figure 1.8: Xyloglucan structure.

Arabinogalactanns

Arabinogalactan has a backbone of β-(1→3)-linked D-galactopyranose units and is highly branched at C6 (Figure 1.9). The side chains are composed of β-(1→6)-linked D-galactose units, D-galactose and L-arabinose units or single L-arabinose units and single D-glucuronic acid units. Arabinose is present in furanose and pyranose forms in an approximate ratio of 1:2. (Sjostrom;E., 1993). Most of softwoods contain only small amounts of arabinogalactan amounting to less than 1% by weight of wood (Shimizu, 2001).

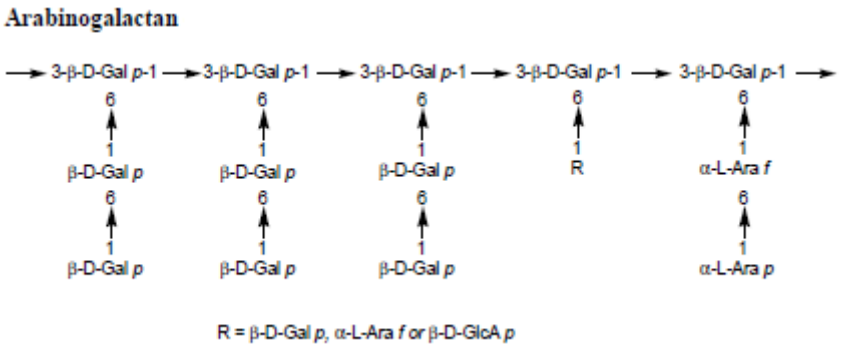


Figure 1.9: Arabinogalactan structure.

1.2.3.Lignin

Lignin is the most abundant aromatic natural polymer and confers a rigid structure, impermeability and resistance against microbial attack and oxidative stress on plants (Goldstein, 1981). It presents a complex hydrophobic, nonlinear, highly branched and randomly linked polyphenolic structure consisted by three main monomeric phenylpropane units, also called as monolignols or monomer alcohols precursors (Figure 1.10A) which are linked by ether bonds. (McCarthy, 2000). *p*-Coumaryl, coniferyl and synapyl alcohols differ structurally from each other by the number of methoxyl groups present on their aromatic ring; they possess zero, one and two methoxyl groups, respectively (Goujon et al., 2003). The proportions of these basic structures vary greatly in different families of plants (Campbell and Sederoff, 1996).

Lignification is the process of polymerization of the monolignols, p-coumaryl, coniferyl and sinapyl alcohols, each, after several hydroxylation and methylation steps of the aromatic ring (Figure 1.10B), in the corresponding methoxylated form as hydroxyphenyl (H), guaiacyl (G) or syringyl (S) lignin units, respectively.

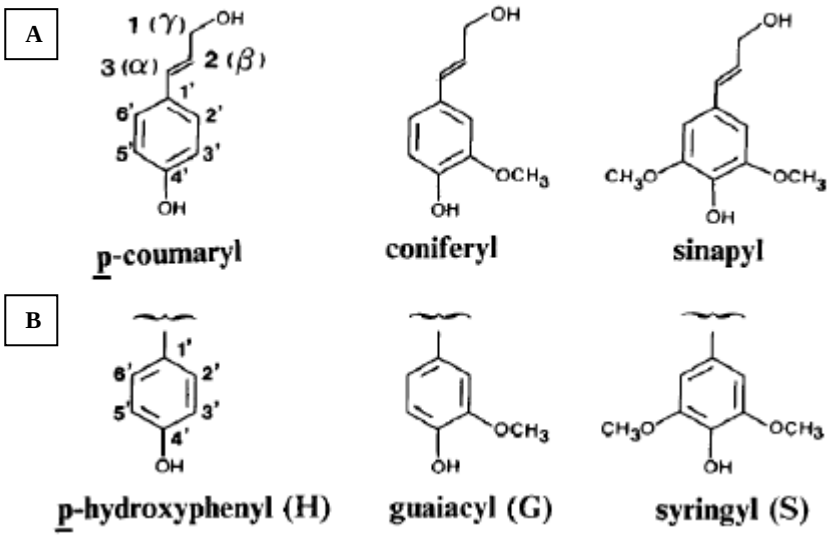


Figure 1.10: (A) Structures of the monomeric phenylpropane precursors of lignin. (B) Corresponding methoxylated forms of phenylpropane precursors of lignin.

Lignin represent 4 - 35% of most biomass composition, 16 - 25% in hardwoods and 23 -35% in softwoods (Bridgewater, 2004). Lignin in softwood is mainly consisted by guaiacyl lignin type containing less sinapyl units whereas syringyl lignins are often found in hardwoods. (Brebu and Vasile, 2010). Softwood lignin generally contains a higher amount of carbon (≈60-65%) than hardwood lignin (≈56-60% carbon) since hardwood contains more methoxy groups which leads to a higher oxygen content (Fengel, D.; Wegener, 1984).

The structure of lignin cannot be represented exclusively using simple monomeric units but rather as building blocks to larger and complex molecules such as dimeric and tetrameric ones where certain linkages and some of the general interunit linkages were already identified. (Palmer, 1983). Lignin units are linked by different bond types within the same lignin macromolecule, however, the most frequent is the β-O-4 ether bond, which is the target of most degradation techniques (Glazer A. W., 1995).

Unlike cellulose and hemicellulose which are uniform chain structures made up of repeating sugar units, polymerization of lignin (i.e., polymerization of the monomers) was originally thought to occur through three-dimensional random coupling reactions (Fengel, D.; Wegener, 1984). However, further studies using computer-assisted structural would later suggest that a helical conformation was more energetically favored over random structures (Faulon et al., 1994). It was proposed by *Fengel et al.* a mechanism for lignin polymerization where dimeric structures would be first formed followed by end-wise polymerization to yield branched aromatic polymers (Fengel and Wegener, 1984) These dimeric structures are suggested to combine to form molecules with DP lower than 20, which then combine to form larger molecules that eventually make up the lignin macromolecule (Wayman and Obiaga, 1974).

A structural lignin model is presented in Figure 1.11 and it only describes a hypothetical structure inferred from the research developed in nearly two decades. Lignin is an amorphous polymer network whose main units are linked to each other by the irregular coupling of ether and carbon-carbon bonds. Therefore, different biomass natures or even lignin that was isolated from the same raw material but using different delignification methods, would lead to a final product with different functional groups composition. Therefore, due to lignin complexity and high heterogeneity, it is extremely complicated to define a lignin model which truly defines its main structure.

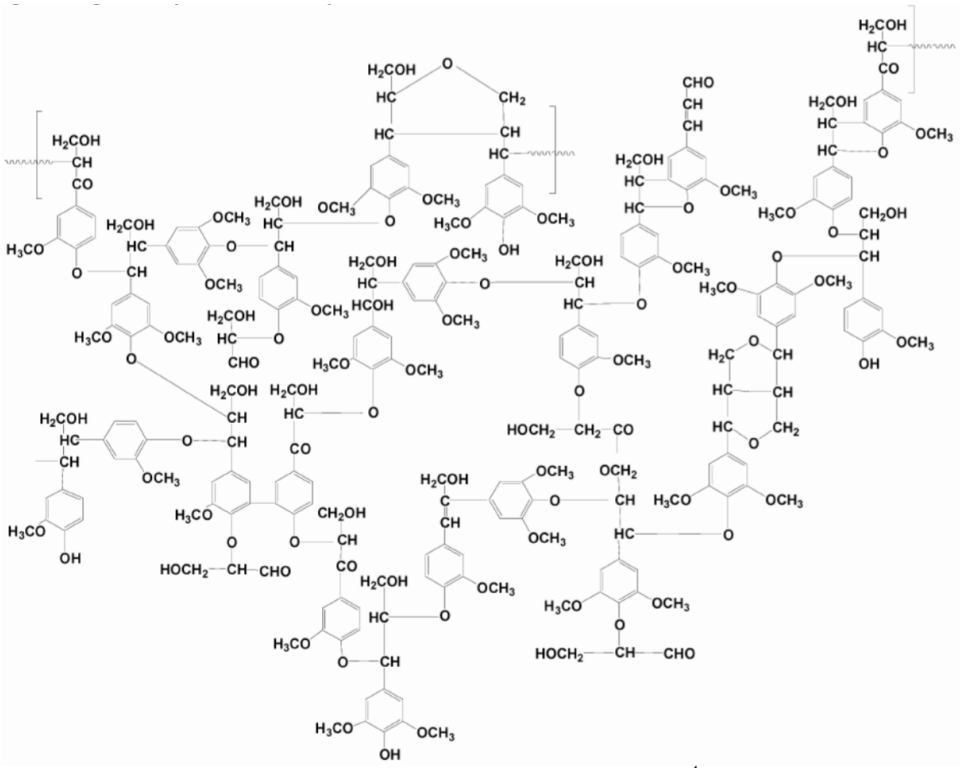


Figure 1.11: Partial structure of hardwood lignin (Mohan et al., 2006).

Due to its complex composition and structure, lignin thermal decomposition occurs over a broad temperature range from 200 ºC to 500 ºC since different oxygen functional groups from its structure have different thermal stability, thus, their scission will occur at different temperatures (Brebu and Vasile, 2010; Saeed, A.; Fatechi, P.; Ni, 2014; Wahyudiono et al., 2013).

1.3. Biomass valorization: Added value by-products

Biomass can be categorized in four main groups: *agriculture*, *forest*, *municipal solid wastes* and *others* which includes fast-growing plants, short-rotation crops, herbage plants and ocean biomass (Cuiping et al., 2004). Main biomass component proportions (cellulose, hemicellulose and lignin) varies largely depending on the type, source and species of the biomass (Pauly and Keegstra, 2008). (Pérez et al., 2002). However, typical biomass, wood and grass plants contains approximately 40-50% of cellulose, 20-30% of hemicellulose, 20-28% of lignin and small percentages of other compounds such as mineral (potassium, sodium, calcium, etc.) and organic extractives such fats, proteins, phenolics, pectines, resins, essential oils, among others (Wu et al., 2008).

Regulatory agencies are increasingly stringent in the application of environmental laws and regulations considering the pollution of agricultural waste streams and the application of toxic solvents during processing in such a way that, nowadays, it has become a public concern. Therefore, there is an urgent need to identify alternative processes capable to treat waste materials properly and at the same time recover valuable products such as phenolic compounds, water soluble sugars, bio-fuels, organic acids and chemicals with valuable applications in pharmaceutical, cosmetics, food industries (Deng et al., 2012; Mussato et al., 2012; Rodrigues et al., 2006).

1.3.1. Carbohydrate applications

The development of a green alternative method for the production of soluble sugars from agricultural residues has captured the interest of many researchers.

Considering bioprocesses, it is generally accepted that the carbon-source represents an average of 30% of the total production cost. For instance, the cost of a standard medium containing 3% (w/v) of glucose is about 50 €/m^3 (Ratledge, C.;Kristiansen, 2006). Considering a production at an industrial scale, it is imperative to reduce this cost to turn the process economically feasible. By using several cheap and readily available agro-residues as an alternative source for carbon, several bio-based products could be produced at a large scale using environmental friendly and competitive bioprocesses (Yang, S; Enshasy, H.E.; Thongchul, 2013).

Large quantities of biomass are generated in agriculture industry such as rice bran, wheat straw, sugarcane bagasse, fruit processing wastes, among others and represents an inexpensive and renewable resource of fermentable sugars for industrial bioprocesses (Viganó et al., 2014). Agricultural waste is composed by cellulose and hemicellulose which can be hydrolyzed into water soluble sugars (Shimanouchi et al., 2014).

Ethanol is produced from several agricultural products such as sugarcane, corn, wheat, sugar beet and cassava, by using their rich polysaccharide structure which, after hydrolysis, will supply, totally or partially, the carbon requirements for the fermentation process (Carbohydrates from Biomass: Sources and Transformation by Microbial Enzymes).

1.3.2. Phenolic compounds

Typically, agro-residues generated during handling and processing of fruits, vegetables or forest resources still contains significant amounts of the original plant components such as fruit skins, fruit seeds, flowers, leaves, stems, barks, and roots. These agro-residues represent alternative sources for several added value natural compounds such as phenolics compounds (Martins et al., 2011; Tahir, 2015). Phenolic compounds are plants secondary metabolites synthesized during plant development as well as in response to different external factors such as stress conditions and UV radiation (Fontana et al., 2013). Its structure comprise an aromatic ring, with one or more hydroxyl substituents and present in a wide range of structures, from a simple skeleton of phenolic acids such as flavonols and catechins and more condensed structures such as tannins with molecular weights of 500 up to 20,000 for proanthocyandins (Balasundram et al., 2006). Most phenolic compounds occur naturally in conjugation with mono- and polysaccharides, linked to one or more phenolic groups or even as functional derivatives, such as esters and methyl esters (Harborne, 1989).

Phenolic compounds are divided into two major classes: **flavonoids**, which are the largest group of plant phenolics and **non-flavonoids** in which phenolic acids are the most studied group (Pandey and Rizvi, 2009).

Flavonoids are low molecular weight compounds with a diphenylpropane (C6-C3-C6) common skeleton which comprises two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Proestos and Komaitis, 2013). According with flavonoids variations within the main structure, they present sub-classes as it is represented in figure 1.12 and it will be further discussed (Rice-Evans et al., 1997).

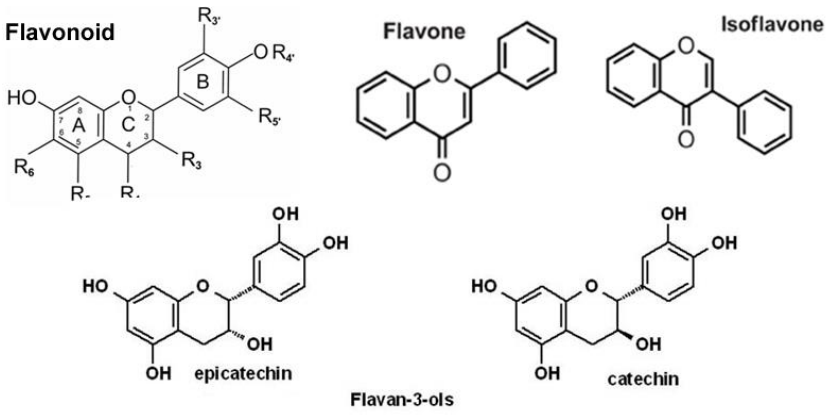


Figure 1.12: Different flavonoids structures. Flavonoids basic structure consists of 2 aromatic rings (A and B rings) linked by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring) (Rice-Evans et al., 1997).

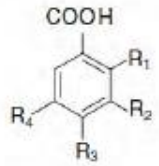
The variations in the phenyl B ring results in the major flavonoid classes:

- Flavonols such as quercetin and kaempferol are mainly found in plants with bright colours, e.g., berries and broccoli (Welch et al., 2008);
- Flavanols or flavan-3-ols including single structures called catechins and multiple-unit structures (dimers, trimers or polymers) such as colorless proanthocyanidins and colorful anthocyanidins (Chai et al., 2015);
- Flavones and isoflavones whose difference is related with the phenyl group location. Isoflavones are mostly found in soy products and the phenyl B ring is located in the 3 position on the C ring (Tsao, 2010);
- Flavanones, mostly found in fresh fruits and their hand-squeezed or industrially processed juices (Gattuso et al., 2007);

Flavones and flavonols are the most widely occurring and structurally diverse flavonoids (Balasundram et al., 2006).

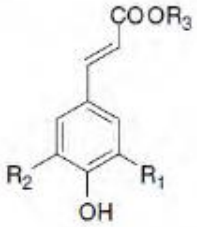
Non-flavonoids includes **phenolic acids** (C6-C1 skeleton comprising a phenolic ring and an organic carboxylic acid function) such hydroxybenzoic and hydroxycinnamic acids, **stilbenoids** (hydroxylated derivatives of stilbenes with a C6-C2-C6 structure) such as resveratrol and **lignans (**consisted of two phenylpropanoid moieties connected via their C8-side chain carbons) (Umezawa, 2003).
As it can be seen in figure 1.13, hydroxybenzoic acids have a C6-C1 structure whereas hydroxycinnamic acids are aromatic compounds with a three-carbon side chain (C6-C3). Further examples from each phenolic acids subclass are identified in figure 1.13(Balasundram et al., 2006).

(A)



Hydroxybenzoic acids	R_1	R_2	R_3	R_4	MW
Gallic acid	H	OH	OH	OH	170
p-Hydroxybenzoic acid	H	H	OH	H	138
Salicylic acid	OH	H	H	H	138
Syringic acid	H	OCH_3	OH	OCH_3	198
Vanillic acid	H	OCH_3	OH	H	168

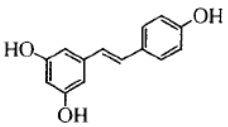
(B)



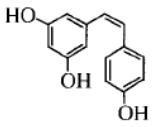
Hydroxycinnamic acids	R_1	R_2	R_3	MW
Caffeic acid	OH	H	H	180
Caftaric acid	OH	H	Tartaric acid	312
p-Coumaric acid	H	H	H	164
p-Coutaric acid	H	H	Tartaric acid	296
Ferulic acid	OCH_3	H	H	194

Figure 1.13: Structures of hydroxybenzoic acids (A) and hydroxycinnamic acids (B) (Moreno-Arribas, M.V.; Polo, 2009).

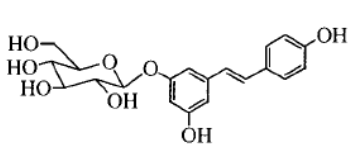
Resveratrol is a stilbenoid and a phytoalexin produced naturally by several plants in response to injury or pathogen attack and it is mostly found in grape skins and seeds, especially in red ones, as well as in peanuts, berries and rhubarb. Also known by 3,5,4'-hydroxystilbene, resveratrol can be found in *cis* or *trans* configurations as well as glucosylated (Figure 1.14). *Trans* isomeric form is the most studied and commonly found in plants (Burns et al., 2002).



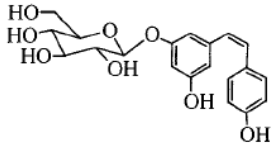
trans-Resveratrol



cis-Resveratrol



trans-Resveratrol-3-*O*-glucoside



cis-Resveratrol-3-*O*-glucoside

Figure 1.14: Resveratrol different structures (Burns et al., 2002).

Recent research has pointed many human health benefits associated with resveratrol consumption. Not only it has a low toxicity in humans and presents a high potential to prevent lipid oxidation but also several studies revealed that resveratrol inhibits the growth of human breast and prostate cancer cell lines, and induces differentiation and apoptosis of myeloid leukemia cell lines (Aggarwal et al., 2004; Kelkel et al., 2010). Moreover, there is a growing evidence of the positive effect of resveratrol

on the prevention or delaying of hearth diseases, ischemic and chemically induced injuries, pathological inflammation and viral infections (Asou et al., 2002).

A wide range of therapeutic properties have been associated to phenolic compounds such as anti-allergenic, anti-inflammatory, anti-microbial, anti-oxidant as reducing agents, inhibiting and delaying lipid oxidation, anti-thrombotic, cardioprotective and vasodilatory effects (Castro-vazquez et al., 2016). It was already demonstrated the relation between the consumption of phenolic compounds and health benefits as well as their emerging role in the prevention of cancer and cardiovascular diseases (Garcia-Salas, 2010). Several studies have been performed about the mechanism of food polyphenols for cancer prevention where it was shown that chemo preventive polyphenols may interrupt or reverse the carcinogenesis process by acting on intracellular signaling molecules network (Link et al., 2010). However, these possible health benefits derived from phenolic compounds would depend on their absorption and metabolism which is directly determined by their structure, conjugation with other phenolics, degree of glycosylation, molecular size and solubility (Balasundram et al., 2006).

1.4. Lignocellulosic residues

The management of wastes, in particular of industrial waste, in an economically and environmentally acceptable routine, is one of the most critical issues facing modern industry, mainly due to the increased difficulties in properly locating disposal places and obeying the extremely stringent environmental quality requirements imposed by legislation. According to modern industrial trends, an eco symbiosis economic efficiency is the key factor for planning modern technological processes, which includes decreasing the waste streams during production and the use of produced waste as by-products or raw materials with higher added values.

Several tones of various lignocellulosic residues are generated every year derived from fruits or vegetables processing as well as from other important industries such as paper or olive oil production which leads to severe pollution problems (Laufenberg et al., 2003). In most of the cases, these residues represent a loss of valuable compounds, which are, usually neglected or used without treatment for animal feed or as fertilizers. In the last few years, however, considering the increasing society interest in preventing environment pollution, economic interests and the need to conserve energy and materials, new methods and new policies for waste handling and treatment have gained interest in order to recover added value compounds from waste materials. Several wastes have a potential for recycling raw materials or for conversion into useful products of higher value as a by-product or even as raw material for other industries (Mussato et al., 2012). During this study, three industrial wastes with a significant impact, were selected to be subject to a valorization process:

recycled paper mill sludge from paper industry, red grape pomace from wine industry and apple pomace from cider industry.

1.4.1. Paper industry: Recycled paper mill sludge

Paper industry is a strategic industry in many countries. However, the production of paper consumes high quantities of energy, chemicals and wood pulp. Consequently, it produces high gases emission levels which are harmful for the environment mainly as CO_2 due to energy consumption as well as high amounts of solid wastes, which include wastewater treatment sludges. According to *Buswell et al.*, 1 ton of low-ash paper mill sludge in landfill releases into the environment approximately 2.69 tons of CO_2 and 0.24 ton of CH_4 (Buswell and Mueller, 1952). In terms of volume, most solids and liquids are originated from the treatment of effluents, although waste derived from primary wood treatment is also produced in large quantities (Likon and Trebše, 2005).

The world's total paper production was 403 million tons in 2013 and is expected to increase 77 % by 2020 (Likon and Trebše, 2005). Asia, which accounts for 45% with 182 million tonnes of paper produced, is the largest paper producer. Europe with a production of 106 million tonnes and North America with 85 million tonnes, are also significant producers.

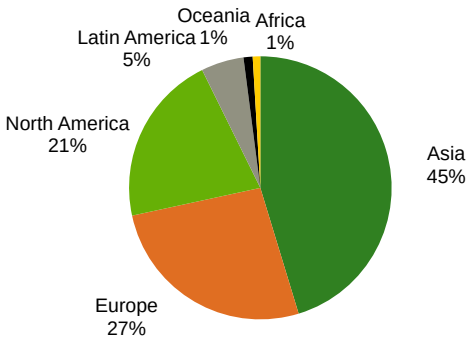


Figure 1.15: Worldwide paper production industry distribution in 2013 (PPI PULP & PAPER).

In terms of market value, global paper industry is expected to reach approximately US $256 billion in 2017 and over 66 % of paper production will be recycled (Hetemäki, 2013) The industry is expected to face certain challenges derived by media extension for digital formats, stricter environmental regulation, volatility in raw material prices, and energy inputs. However, increasing literacy rate, recycling, population and economic growth are anticipated to boost the industry. Moreover, new technologies have been launching new challenges with the increasing demand for specialty papers in

packaging as well as printing applications, which will be the major drivers for the global pulp and paper market in the future (Lucintel, 2012).

Pulp and paper mill wastewater treatments typically include a primary treatment consisting of neutralization, screening, and sedimentation to remove suspended solids, which are then dewatered into a sludge that requires disposal. This solid residue is called paper mill sludge (PMS) and is the major waste generated from paper production and recycling. The production of dry sludge corresponds to approximately 4.3% of the paper-type final product, increasing to 20–40% in the case of recycled paper mills (Smith and Scott, 1995) (Bank, 2007). Often secondary and tertiary treatments are included to reduce the organics content of wastewater and destroy toxic organics and color. Pulp and paper mill primary sludge contains wood fibers as the principal organic component, as well as papermaking fillers, pitch (wood resin), lignin by-products, inert solids rejected during the chemical recovery process, and ash (Ochoa de Alda, 2008).

Figure 1.16 represents a simple flowchart of PMS generation.

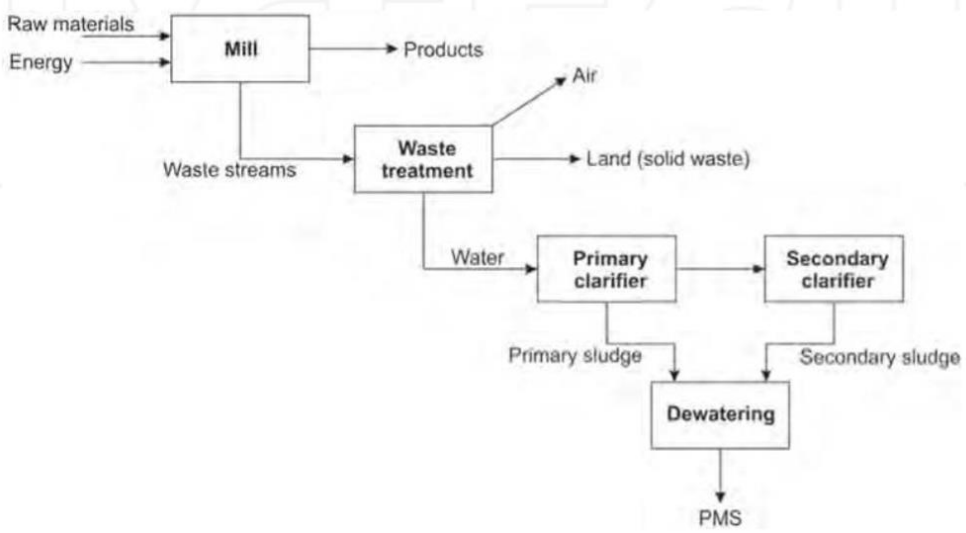


Figure 1.16: PMS generation flowchart (Likon and Trebše, 2005).

The countries joined in the CEPI organization (Confederation of European Paper Industries which includes the European pulp and paper industry) produce more than 4.7 million tons of PMS per year and global production of PMS was predicted to increase between 48 and 86% above the current level, in the next 50 years. This represents an enormous environmental burden considering that more than 69 % of the generated PMS is landfill disposed (Mabee,W.; Roy, 2003).

Wastewater purification in pulp and paper mills combines sedimentation, biological treatment, chemical precipitation, flotation and anaerobic treatment, and the specific combination of techniques is determined by the local conditions (Figure 1.17). PMS characteristics depends on pulp and paper

mill processing as well as on the raw materials used. PMS can be divided into two main types: high-ash sludge (> 30% dry weight) and low-ash sludge (< 30% dry weight). High-ash sludges are chemical flocculation sludges generated by pulp mills, primary sludges generated by production of paper from recycled fibers and deinking sludges generated by paper mills, alternatively, low-ash sludge represents primary, secondary or biological sludges generated by pulp or paper mills.

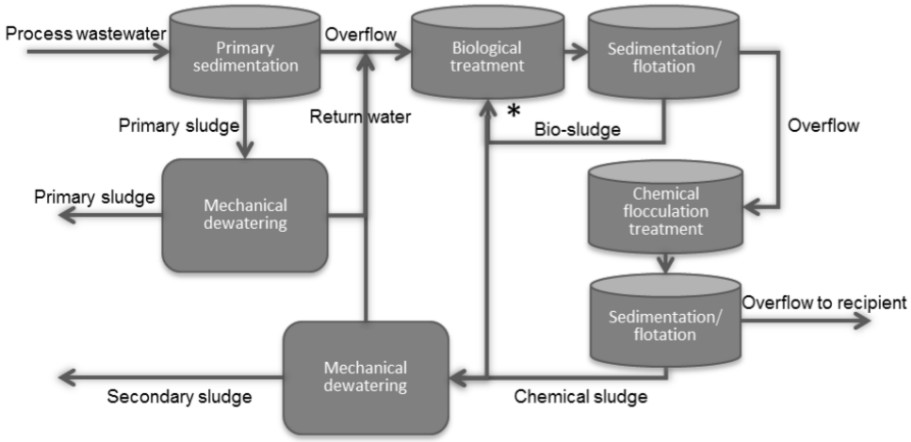


Figure 1.17: Schematic flow chart for wastewater treatment during paper production (Hagelqvist, 2013).

Currently, most PMS is dried, spread or deposited onto the landfill (Likon and Trebše, 2005). As an alternative to landfill, PMS is incinerated on the sites for energy recovery, but the economics of incineration is questionable because PMS contains 30 to 50 % of water and for each additional 1 % of moisture content in PMS, the temperature of combustion must be 10°C higher due to process efficiency (Kraft, D.L.; Orender, 1993).

Another important source of fiber for papermaking is recycled paper, which is rewetted and reduced to pulp after removing inks, adhesives, and other contaminants by chemical deinking and mechanical separation. Recommended wastewater reduction methods include the recycling of wastewater with simultaneous recovery of fibers (World Bank, 2007). The most common technique for reclaiming fiber is to recycle primary sludge back into the fiber-processing system of the mill. However, recyclers are producing two to four times more sludge than virgin pulp mills producers, as already mentioned earlier. Increased recycling over the past two decades has consequently increased the amount of material that requires disposal (Likon and Trebše, 2005). Appropriate managing of such an amount of waste is the most crucial task for modern pulp and paper industry.

Figure 1.18: Recycled paper mill sludge (Abdullah et al., 2015).

PMS chemical composition it strongly depends on the paper grade manufactured, specific fresh water consumption, the wastewater cleaning technique applied and the type of raw materials (e.g. wood, fillers). Therefore, the chemical composition of paper mill sludge produced is often significantly different between mils (Battaglia et al., 2003; Jackson and Line, 1997). The main organic components in the paper mill sludge are wood, cellulose fibers of different lengths and lignin. The main inorganic components are kaolinite (clay) and calcium carbonate, which are paper additives, as well as heavy metals present as impurities, which are mainly originated from the wood raw material (Zhang et al., 2004). Inorganic components are usually predominant in sludge from printing paper and board production, whereas in the packaging paper industry sludge a more organic character is typical (Kuokkanen et al., 2008). Distinct differences in chemical composition between virgin and recycled mills are observed as well as significant variations within the same categories. In table 1.1, it can be observed elementary analysis of different sludges produced in paper industry from different origins.

Table 1.8: Paper sludge elementary analysis produced from different sources (value of wastepaper for comparison). In the Kraft unit, it occurs the breakage of bonds between lignin and cellulose. In the deinking unit, it is processed the printing ink removal form paperfibers of recycled paper (Smith and Scott, 1995).Two different deinking mill units are represented in table by (1) and (2).

	Analysis (%)							
Source	Solids	Ash	C	H	S	O	N	Heating value (MJ/kg)
Wood chips	79.5	0.2	49.2	6.7	0.2	43.6	0.1	19.4
Pulp mill	42	4.9	51.6	5.7	0.9	29.3	0.9	21.5
Kraft mill	37.6	7.1	55.2	6.4	1.0	26.0	4.4	24.1
Bleached pulp mill	33.4	1.9	48.7	6.6	0.2	42.4	0.2	20.1
Recycled paper mill	50.5	2.8	48.6	6.4	0.3	41.6	0.4	20.6
Deinking mill (1)	42.0	20.2	28.8	3.5	0.2	18.8	0.5	12.0
Deinking mill (2)	42.0	14.0	31.1	4.4	0.2	30.1	0.9	12.2
Wastepaper	92.0	7.0	48.7	7.0	0.1	37.1	0.1	25.0

In table 1.1, it can be observed the differences between chemical compositions of sludges both obtained from deinking units, mainly in ashes content. In general, paper mill sludge is highly fibrous while Kraft pulp mill sludge tends to be higher in sulfur compounds. Deinking mill sludge tends to have high ash levels, depending on the type of recycled paper used. High ash is related with a sludge lower heating value. For instance, the high-ash sludge has only 60% of the heating value of the low-ash sludge. This significantly affects its suitability for incineration (Smith and Scott, 1995).

An increase in the number of management alternatives would improve the feasibility of reusing paper sludge in a more sustainable manner. Several new procedures have been studied and supercritical water treatment of pulp and paper mill sludge has been pointed as a promising alternative to use paper sludge to produce high quality fuels, such as, methane, hydrogen and heavy oils from wet sludges (Bajpai, 2015; Zhang et al., 2010). This technique is especially interesting because it does not require extensive dewatering beyond what can be achieved with mechanical dewatering. Some of the products may also be useful as replacements for fossil based oils in incinerators (Guan et al., 2016; Hagelqvist, 2013). In addition to supercritical water conditions, hot compressed water was also evaluated to treat secondary pulp and paper sludge for glucose recovery where 90 mg/L of hydrolyzed glucose was obtained with HCW treatment performed at 220ºC during 20 min. (Chariyaprasertsin et

al., 2013). Due to the high carbon concentrations, several studies were performed to enzymatically hydrolyze paper sludge into smaller carbohydrates to be further assimilated by microorganisms (Elliston et al., 2013; Guan et al., 2016; Kumar et al., 2016). For instance, *Pichia stipitis* was used to produce ethanol by using RPS as carbon source and after 48 h of incubation, a production of 18.6 $g.L^{-1}$ of ethanol from 178.6 $g.L^{-1}$ of dried RPS was obtained corresponding to an overall conversion yield of 51% of the available carbohydrates on the initial substrate (S. Marques et al., 2008). *Saccharomyces cerevisiae* was also applied for the production of bioethanol (Peng and Chen, 2011) with a conversion rate of sugar to ethanol of 34.2% and an ethanol yield of 190 $g.kg^{-1}$ of dry paper sludge, corresponding to an overall conversion yield of 56.3% of the available carbohydrates on the initial substrate. Lactic acid was also produced by *Lactobacillus rhamnosus* with 73 $g.L^{-1}$ of lactic acid production, corresponding to a maximum productivity of 2.9 $g.L^{-1}.h^{-1}$, with 0.97 g LA produced per g of carbohydrates on initial substrate. (Susana Marques et al., 2008). These studies demonstrated the feasibility of the biological conversion process of paper sludges into added value products under a multi-purpose biorefinery concept. Further details about HCW technology will be discussed later in this chapter.

1.4.2. Wine making industry: Red grape pomace

Grapes, mainly the fruiting berry of woody vines belonging to the *Vitis vinifera* species, are one of the most abundant world fruit crops with a yearly production of more than 70 million tons (Fontana et al., 2013). Currently, there are between 5.000 and 10.000 varieties of *Vitis vinifera* grapes though only a few contain commercial significance for wine and table grape production (Trust, 2012).

According with the report from the International Organization of Vine and Wine (OIV) in 2014, over 50% of the world´s vineyards was mainly distributed by 5 countries, namely, Spain (14%), China (11%), France (10%), Italy (9%) and Turkey (7%). Portugal occupied the 8th place. Europe was the highest grapes producer with 41% of world grapes, followed by Asia with 29% and 21% in America. In China almost all grapes are used as fresh grapes, while in European countries, namely France, Italy and Spain, and in the U.S., grapes are mostly used for wine production. In 2015, global wine production was estimated to have increased by 2%. Initial estimates presented predict total wine production in 2015 of 275.7 million hectoliters. Italy saw the biggest growth producing 48.9 million hectoliters. Italy was followed by France, which produced 47.4 million hectoliters, an increase of just 1% compared with 2014. Portugal and Romania increased their production by 8% and 9% respectively, rising to 6.7 and 4.1 million hectoliters.

Wine making industry relies on a complex process where traditions and secrets are, most of the times, kept by their wine makers turning wine processing not only a chemical process but also a kind of art.

Basically, wine is the product of total or partial alcoholic fermentation of fresh grapes with a minimum alcohol percentage of 8.5%. There are two main methods for wine making, which are white and red methods. In the white method, grapes are firstly smashed, the grape juice decanted which then goes through the fermentation process, while in the red method the seeds, skins and pulp are removed only after fermentation in order to maintain grapes pigments and tannins in the wine. Red wine is made through the red method whose fermentation must contains grape solids components (seeds, skins and sometimes stems) and their sugars conversion into alcohol and carbon dioxide by yeasts usually takes place in stainless steel vessels or wooden fermentation tanks. The resultant red grape pomace, the residue generated during pressed after fermentation, contains low amount of soluble sugars and has already lost many of its tannins and colored compounds to the wine.

It has been estimated that an equivalent amount of 18–20% of the grapes used during wine making process is generated as by-product, which could easily originate more than 13Mton (fresh weight) of waste biomass per year (Spanghero et al., 2009). Currently, grape pomace is considered to have limited economic value being used for the preparation of alcoholic beverages, cattle feeding, soil conditioning and as fertilizer. However, the main percentage is dumped in disposal sites. This represents high costs for the winery industry since, if not treated, it can create several environmental hazards (Arvanitoyannis et al., 2006).

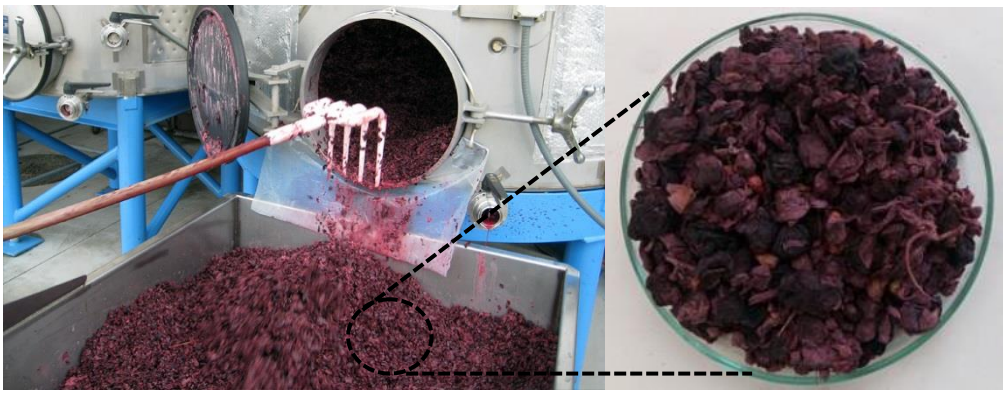


Figure 1.19: Red grape pomace.

Recently, grape pomace potential has been investigated for several industrial applications such as nutritional ingredient isolation, dietary fiber, citric and tartaric acids production, grape seed oil, natural food colorants and compounds for therapeutic use, such as phenolics (Dwyer et al., 2014; Fontana et al., 2013; Mendes et al., 2013). It has also been proposed as a raw material for bioenergy production (Toscano et al., 2013) such as bioethanol, biobutanol and biogas (Cáceres et al., 2012).

Grape pomace consists of water, proteins, lipids, carbohydrates, minerals, and compounds with important biological properties such as fiber, vitamin C, and phenolic compounds (tannins, phenolic

acids, anthocyanins, and resveratrol), depending on the type of waste, the cultivar and climatic and cultivation conditions (Sousa et al., 2014). It is very important to highlight that the physical-chemical properties of the final residue are closely dependent on the specific vinification procedures, which consequently, determine its further use and specific valorization circuit in which it could be integrated.

Grape marc, consisting in skin and pulp, is the major components of grape pomace representing more than half of the material weight, being the remaining part grape stems (Mendes et al., 2013). Red grape pomace composition differs largely according with the type and variety of grape used. The average chemical composition of the three most used red grape varieties, which are Cabernet Sauvignon, Pinot Noir and Merlot, are presented in table 1.2.

Table 1.9: Average chemical composition of red grape pomace (Dwyer et al., 2014; Teixeira et al., 2014).

Component	% (on dry matter basis)
Structural constituents	
Skin	60-80
Seed	14-20
Stems	0.5-2
Chemical composition	
Cellulose	27-37
Hemicellulose	37-54
Lignin	16.8-24.2

Grape skins are composed of cellulose, hemicellulose, pectin and lignins arranged in a complex matrix and represent the main grape pomace component with higher amounts of phenolic compounds. Although 30 - 40% of the phenolic compounds are extracted during the vinification process depending on grape varieties, vineyard location and technological parameters of wine making (destemming, crushing, maceration and pressing), grapes are still extremely rich in these bioactive compounds. Considering the extractable phenolic compound fraction, grape pomace is rich in anthocyanins, catechins, procyanidins, flavonol glycoside, phenolic acids, and stilbenes with great emphasis on resveratrol. Grape pomace also contains high amount of non-extractable polyphenols, such as polymerized condensed tannins and some polyphenols that form complexes with fiber (Yu and Ahmedna, 2013). Average chemical composition of grape skins can be observed in table 1.3.

Table 1.10: Grape pomace skins average chemical composition (Dwyer et al., 2014).

Skin Component	% (on dry matter basis)
Soluble sugar	1.4-1.7
Total dietary fiber	51-56
Insoluble dietary fiber	49.5-55
Soluble dietary fiber	0.8-1,7
Phenolics	
Condensed tannins (% dry matter)	16-20
Total Phenolic Content (mg GAE/g dry matter)	11-27

Other products of interest from grape pomace are located in grape seeds which are a complex matrix containing approximately 40% fiber, 13.1 – 19.6 % (v/w) of oil content, 11 % proteins and 7% complex phenols including tannins in addition to sugars and mineral salts (Dwyer et al., 2014; Nair, S.; Pullammanappallil, 2003).

Grape seeds contain about 8 - 22% edible oil, marketed as low cholesterol frying oil (Nair, S.; Pullammanappallil, 2003) with 86% of unsaturated fatty acids. The major fatty acids of grape's seed oil are linoleic acid (66.7-73.6%), oleic acid (17.8-26.5%), palmitic acid (6.35-7.9%) and stearic acid (3.64-5.26%) (Teixeira et al., 2014) (Lvarruiz and Ardo, 2009). Grape seed extracts have been reported to have several pharmacological applications, most of them due to the presence of phenolic compounds. Grape seeds also contains non-phenolic antioxidants such as tocopherols and β-carotene both compounds with potent antioxidant activity and with crucial human health benefits (Bordiga, 2016).

Considering red grape pomace potential, attention has also been paid to the recovery of sugars (Fontana et al., 2013). Several recent technologies were applied for the extraction of added value compounds such as hot compressed water extraction defined as an extraction technique that uses liquid water as extractant (extraction solvent) at temperatures above the atmospheric boiling point of water but below the critical point of water. Applying temperatures of 80–175°C and short extraction times, HCW extraction was defined as a "greener", faster and more efficient technique over conventional extraction methods (Plaza and Turner, 2015). This topic will be further discussed later in this chapter.

1.4.3. Cider industry: Apple pomace

Cider is an alcoholic beverage made from the fermented juice of apples. Although the cider industry is considerably smaller than the wine, beer, or spirits industries, it is a fact that in recent years in some EU countries, cider and fruit wines have enjoyed one of the fastest growth rates of all alcoholic beverages. There are many hundreds of apple varieties developed specifically for cider making. Apples grown for consumption are suitable for cider making, though some regional cider-makers prefer to use a mix of eating and cider apples or exclusively cider apples. In order to obtain the accurate cider, these apples must be juicy, sweet, well ripened and have adequate levels of natural acids and tannins.

Cider is very popular in the United Kingdom with the world's highest per capita consumption, as well as it is the largest cider-producing country with an annual production of more than 370 million litters (AICV, 2014). Cider is also popular in other European countries including Ireland, Portugal (mainly in Minho and Madeira), France, Spain, Germany and Switzerland. Although smaller cider markets also exist in other countries, cider is still a mainly European product.

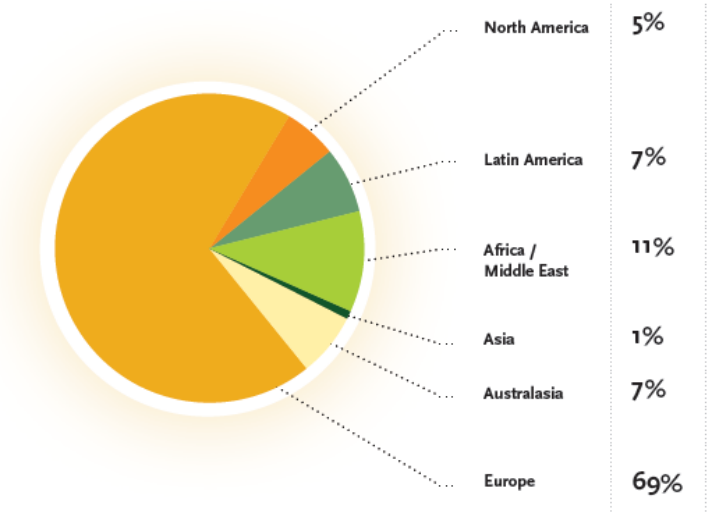


Figure 1.20: Cider industry distribution worldwide (AICV, 2014).

The cider making process typically involves three stages: crushing the fruit, pressing out the juice and fermentation. Firstly, fruit is harvested, sorted, and washed. In contrast with the wine industry where the grapes need only a light crushing to break the skins before releasing the juice, apples must first be milled to a pulp before the juice can be pressed out. This is done to ensure that the maximum amount of juice can be extracted from the apples. In order to remove the juice, the resulted pulp is then pressed. The apple juice is expelled from the pomace and allowed to ferment.

Nevertheless, it is important to emphasize that apples are not only used for cider production but it is actually the fourth most widely produced fruit in the world after bananas, oranges and grapes.

Considering worldwide apple production, 71% of the fruit is consumed for table purposes as fresh fruit. About 20% of apple production is industrially processed from which 65% are processed into apple juice concentrate, generating large amounts of solid apple pomace (Shalini and Gupta, 2010).

Apple pomace is the solid processing waste generated after apple juice manufacturing and consists of a complex mixture of peel, core, seed, calyx, stem, and soft tissue, representing up to 30% of the original fruit (Vendruscolo, F.; Albuquerque, P.M,; Streit, F., Esposito, E.; Ninow, 2008). Apple pomace is a by-product generated not only by cider industry but also from several other industries such wine, brandies production, spirits and vinegars industries (Sato et al., 2010).

Figure 1.21: Apple pomace.

Apple pomace is considered as a poor animal feed supplement due to several reasons: (1) a high lignin/cellulose ratio with a low digestibility, (2) low nutritional level due to the very low vitamin and mineral content, (3) a high free sugar content, which after ingestion by ruminants and fermentation in the rumen causes animal alcoholaemia (Villas-boas, 2003). Due to the high sugars concentration, apple pomace has a great potential for biotechnological processes acting as a carbon source for microorganisms (Vendruscolo, F.; Albuquerque, P.M,; Streit, F., Esposito, E.; Ninow, 2008). Apple pomace is rich in both soluble carbohydrates such as simple sugars (fructose, glucose, sucrose) and polysaccharides (cellulose, hemicellulose, pectin) (Parmar and Rupasinghe, 2013). Several studies were reported using the conversion of apple pomace into soluble sugars for citric acid production (Xie and West, 2006), lactic acid (Gullón et al., 2008), enzymes (Berovic and Ostrovers, 1997), ethanol and acetic acid (Parmar and Rupasinghe, 2013). Moreover, pectin can be also recovered and used as a gelling agent, stabiliser and source of dietary fiber (Reis et al., 2014).

In addition to carbohydrates, apple pomace has been also presented as a rich source of polyphenols and some phenolic constituents, especially procyanidins and quercetin glycosides. The main polyphenols are present in apple peel and include catechin, epicatechin, hydroxycinnamate, phlorentin glycoside, quercetin glycoside, procyanidin, chlorogenic acid and phloridzin (Chandrasekaran, 2012).

Polyphenolics from apple pomace are considered highly valuable compounds which may be used as functional food ingredients and as natural antioxidants to replace their synthetic equivalents.

Once again, apple pomace chemical composition strongly depends on the type of apple used, storage conditions, pressing procedures, among others factors. Apple pomace is considered an unstable residue not only due to its physicochemical composition but also due to the presence of enzymes activated after plant tissue disintegration (Sato et al., 2010). Table 1.4 presents an average of the chemical and physical composition of apple pomaces collected from eleven different cultivars in Brazil.

Table 1.11: Average chemical composition of apple pomace (Sato et al., 2010).

Component	Content
Moisture (%)	11.43
Ash (%)	1.80
Lipids (%)	1.53
Proteins (%)	2.74
Total polyphenols (g/kg)	4.61
Total sugar (g/100g)	39.35
Dietary fiber (g/100g)	43.63

Considering this, apple pomace represents a highly available residue with a great potential for the production of added-value compounds through the recovery of its main components, mainly sugars and phenolic compounds.

1.5. Biomass conversion conventional methods

As aforementioned, biomass presents many advantages, comparing with other renewable resources, as a promising candidate to take place of fossil fuels and other added value compounds in the future. However, biomass does not seem to be suited for direct combustion with fossil fuels since it has a low caloric value, low energy density, high volatile content and high hydrophobicity which turns biomass into a low-grade fuel (Wannapeera and Worasuwannarak, 2012). On the other hand, biomass rigidity needs to be overcome since it represents physical barriers to mass transport not allowing added value compounds recovery from biomass main structure (Isaac et al., 2015). Therefore, a pretreatment is necessary to upgrade its fuel properties or to simplify biomass conversion into other valuable chemicals (Wannapeera and Worasuwannarak, 2012). An appropriate pre-treatment has to be able to (1) disrupt hydrogen bonds in crystalline cellulose and increase its porosity and surface area, (2) break down the cross-linked matrix of hemicellulose and lignin, (3) avoid the degradation or loss of carbohydrate and result in (4) an efficient and economical competitive hydrolysis process (Kumar et al., 2009).

Nowadays, the most important and available techniques for biomass conversion into sugars, mainly from cellulose and hemicellulose structures, can be divided into thermochemical or biochemical processes. Regarding the thermochemical route, two types of hydrolysis is considered: acid hydrolysis as the most applied method and alkaline hydrolysis (Bobleter, 1994). Acid hydrolysis can be further classified as dilute acid hydrolysis (Chandel, 2012), or concentrated acid hydrolysis (Wijaya et al., 2014) For the biochemical route, enzymatic hydrolysis is often performed for biomass pretretament (Sun and Cheng, 2002).

Acid hydrolysis is the traditionally and mostly used technology in industrial applications (Sun and Cheng, 2002) and corresponds to lignocellulosic materials carbohydrates recovery due to the action of diluted acidic solutions at high temperatures or when moderated temperatures are used along with concentrated acidic solutions. The most commonly acids used are sulfuric, hydrochloric or hydrofluoric acids or mixtures of inorganic with organic acids, such as formic acid (Kumar et al., 2009).

Concentrated acid hydrolysis process for the production of fermentable sugars was firstly reported at 1883 where the ability of concentrated sulfuric acid to dissolve and hydrolyze native cellulose in cotton was demonstrated (Harris, 1949). Concentrated acids, such as H_2SO_4 and HCl, are applied to disrupt the hydrogen bonds between cellulose chains and convert the crystalline cellulose structures into amorphous ones which are then diluted in water, at lower temperatures, and the decrystallized cellulose could be completely and rapidly hydrolyzed to glucose with little degradation. This process can achieve the theoretical glucose yield of 90%, but the concentrated acids used (between 1-30% and often even as higher as 75%) are toxic and hazardous and have strong corrosive effect on the reactor

(Binod, P.; Janu, K. U.; Sindhu, R.; Pandey, 2011). Moreover, a large amount of toxic waste stream is generated during neutralization of the acidic solution. The hazards of handling concentrated acids and their recycling complexity have limited the adoption of this technology at industrial scale.

Dilute acid processes were industrialized in the early part of the 20th century (Torget et al., 2000). Diluted acid hydrolysis process usually employs sulphuric acid or hydrochloric acid at concentrations of 2-5% and high temperatures in the range of 120–230 °C (Barta et al., 2010)(Kumar et al., 2009). This process was more advantageous than concentrated acid hydrolysis due to the fact that acid neutralization was easier, eliminating the complex downstream processing as well as equipment and production costs were also significantly reduced. However, it also has many drawbacks mainly a low sugar yield and the formation of by-products from the thermal decomposition of sugars under the high temperatures needed (Yang, 2015). During dilute acid hydrolysis, lignocellulosic biomass is converted to sugars, which may be further degraded to other products, typically furfural. Hemicellulose-derived sugars degrade more rapidly than cellulose-derived sugars (six-carbon sugars). In order to decrease sugar degradation and maximize the sugar yields during biomass hydrolysis, a two-stage process could be developed where the first stage is operated under mild conditions to recover five-carbon sugars, while the second stage is optimized to recover six-carbon sugars (Wu et al., 2008).

Alkali lignocellulosic pretreatment can also be used and applies milder conditions than other pretreatment technologies. The process can be carried out at ambient conditions and, comparing with acid processes, it causes less sugar degradation and many of the caustic salts can be recovered and/or regenerated. However, the pretreatment times are much higher than in acid hydrolysis, normally in the order of hours or days (Mosier et al., 2005).

Biological treatments are gaining researchers and industries interest as a safe and environmental friendly process for lignocellulosic residues hydrolysis. Enzymes can be used to depolymerize biomass producing sugars or other value-added chemicals; for instance, cellulases are often used to break down cellulose into its constituent sugars (Modenbach and Nokes, 2013). Enzymes used during enzymatic hydrolysis should be highly specific and the process is always conducted at mild conditions, normally at a relatively low pH and a temperature range from 45 to 50 °C (Duff and Murray, 1996). Comparing with thermochemical processes, enzymatic hydrolysis has the main advantages of eliminating acid/base recovery and/or neutralization steps as well as excluding equipment corrosion issues. However, it is extremely time-consuming with an hydrolysis rate considerably low in most biological pretreatment processes (Kumar et al., 2009). Moreover, enzymatic hydrolysis final products should be removed as soon as they are formed since high concentrations of some of those by-products can inhibit enzyme activity, which further leads to the low efficiency of hydrolysis process. Compounds generated or released during pretreatment of lignocellulosic biomass can affect

cellulolytic enzymes mainly cellobiose, to a certain extent also glucose, furan derivatives, weak acids and phenolic compounds derived from sugars. Gluco- and xylo-oligosaccharides also have inhibitory effects on cellulolytic enzymes (Koppram et al., 2014; Tsai and Meyer, 2014). Their inhibition mechanism is not only based on the inhibitory effect caused by each compound individually, but also on their interactions (synergy) (Koppram et al., 2014). Additionally, enzyme production as well as enzyme recovery makes this process economically unfeasible at larger scales (Binod, P.; Janu, K. U.; Sindhu, R.; Pandey, 2011).

The key challenge is to develop hydrolysis technologies that are capable of recovering sugars effectively and efficiently. Supercritical fluids have been reported as an alternative and promising treatment method to extract added value compounds as well as to break lignocellulosic structure (Matsumura et al., 2006a; Ruiz et al., 2011). Hot-compressed water (HCW) or subcritical water is a highly promising, energy efficient and environmentally benign solvent to be used for biomass treatments (Möller et al., 2011). Due to its physical and chemical unique properties, hot compressed water may be used as both a solvent and reactant simultaneously enhancing biomass hydrolysis (Wu et al., 2008).

1.6. Hot compressed water

The interest on supercritical fluids technology emerged in the late 70's stimulated by several events such as the first oil crisis and environmental concerns (Brunner, 2009).
The critical point of a substance consists on the endpoint of its gas-liquid equilibrium curve. A supercritical fluid is defined as a substance above its critical temperature and pressure acquiring particular proprieties such as a gas-liked viscosity and liquid-liked density (Nalawade et al., 2006). In addition, slight changes in temperature and pressure near the critical point have a significant impact on fluid density allowing the manipulation of the supercritical fluid proprieties and tuning the process for the dissolution of the target product (Wen et al., 2009).

Supercritical carbon dioxide (CO_2) is the most common supercritical fluid since it is chemically inert, non-flammable and with a low toxicity (Budisa and Schulze-Makuch, 2014). Nowadays, it attracts attention from research and industrial communities, mainly for bioactive chemicals extraction processes. Supercritical carbon dioxide is a good solvent for many non-polar and some polar low molecular weight compounds (Nalawade et al., 2006). But to dissolve highly polar compounds, higher pressures or co-solvents are necessary (Otero-Pareja et al., 2015).

Sub-critical water, also called superheated water, pressurized water and henceforth designated as Hot Compressed Water (HCW), is defined as water in the liquid state and temperatures between the boiling point and the critical point (374ºC and 218 bar), under pressurized conditions (Figure 1.22)

(Abdelmoez, W.; Abdelfatah, R.; Tayeb, A.; Yoshida, 2011). HCW treatment technology is gaining relevance and interest within scientific community as a promising method able to recover added value products from waste materials.

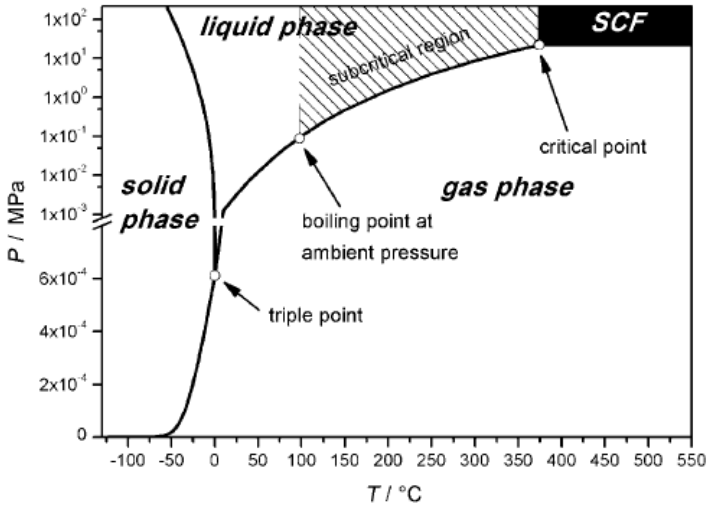


Figure 1.22: Water phase diagram and sub-critical region identification (Möller et al., 2011).

Due to the fact that it is a non-toxic solvent, non-flammable, inexpensive and readily available, HCW is considered as a promising truly ecofriendly technology (Abdelmoez, W.; Abdelfatah, R.; Tayeb, A.; Yoshida, 2011). The unique proprieties of HCW and supercritical water stimulated the research development in several fields mainly, on alternative fuels, biomass conversion and waste disposal (Brunner, 2009).

Solvent properties are physically determined and strongly dependent on temperature and pressure which are variables that can be adjusted according to the process requirements. Consequently, temperature and pressure variables have a direct impact in the reaction, mainly in activation energy and reaction equilibria (an increase of process temperature, increases the thermal energy promoting higher reactions rates), as well as an indirect reaction impact, mainly in solvent proprieties (Möller et al., 2011). Temperature is the variable with the strongest reaction impact whereas most of HCW physical proprieties are weakly dependent on pressure conditions.

At near critical or supercritical conditions, water presents specific physical-chemical properties of which the most important ones will be described below.

HCW has a lower viscosity and surface tension near the critical point comparing to room conditions, which increases mass transfer rates promoting chemical reactions limited by mass-transfer (Möller et

al., 2011). Near the critical point, diffusion coefficient increases at least one order of magnitude and gases such as O_2, N_2, CO_2 are completely miscible (Brunner, 2009). Between ambient and supercritical conditions, water modifies its characteristics from a solvent for ionic species to a solvent for non-ionic species, which can also represent a process drawback when processing residues, since salts dissolved in the feed stream may precipitate and eventually causing reactor corrosion which is more pronounced with the increase of temperature (Möller et al., 2011). Comparing with room conditions, water pH-value can decrease by 3 units promoting acid catalyzed reactions due to a high amount of hydronium ions availability (Brunner, 2009).

Considering the present work, HCW possesses two main characteristics, which are very important for the main goal of this study, which is the lignocellulosic material hydrolysis and valorization: a lower dielectric constant and a higher ionic product when comparing to water at room conditions.
A lower dielectric constant due to hydrogen bond dissociation results in the ability of water to act as a solvent for compounds with higher hydrophobicity (Gonçalves et al., 2009). This promotes the extraction of compounds that are normally extracted with organic solvents such as ethanol or methanol. At room conditions, the dielectric constant of methanol and pure water are 32.6 and 78.5, respectively (Singh and Saldana, 2011). From figure 1.23, it can be observed that when liquid water is heated to 225 ºC, water dielectric constant decreases reaching the value of the dielectric constants of methanol (Amashukeli et al., 2007).

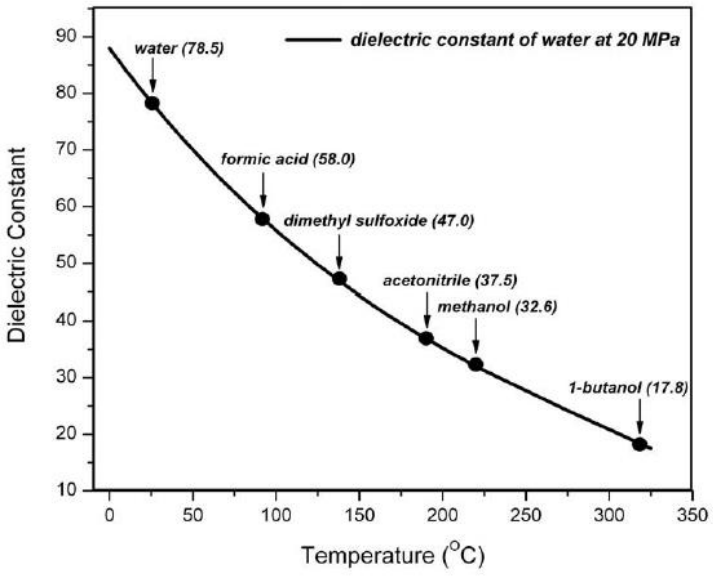


Figure 1.23: Water dielectric constant as a function of temperature at a constant pressure of 20 MPa (Amashukeli et al., 2007).

Figure 1.24 represents water ionic product behavior in sub-critical region and it can be observed that the ionic product increases by increasing of the reaction temperature reaching a value of pK_w of

around 11 at 300 ºC, three times higher than pK_w of water at room temperature. The higher the ionic product, the higher is water dissociation leading to an increase of H^+ e OH^- medium concentrations. This allows water to behave as an acid and/or base catalyst promoting hydrolysis (Carr et al., 2011). At higher temperatures, the ionic product starts to decrease to values of pK_w below 20.

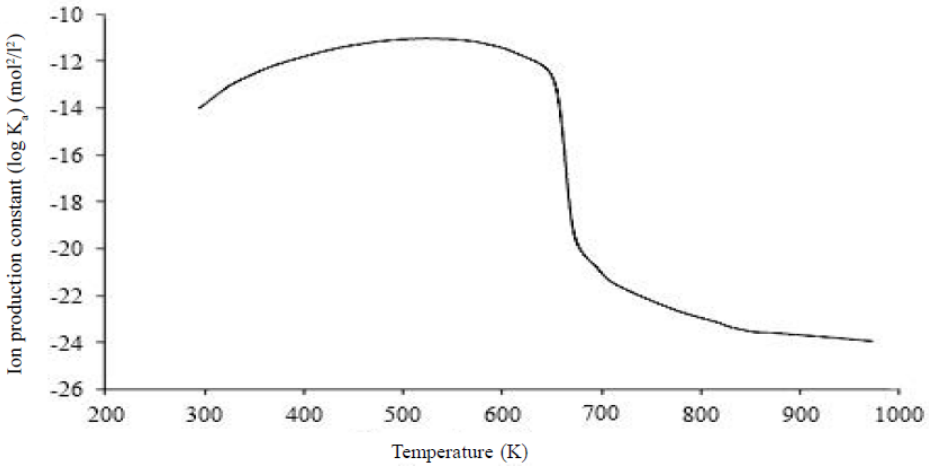


Figure 1.24: Temperature effect on water ionization constant (Sub-critical water as a green solvent, 2015, Shitu).

Table 1.5 summarizes water physical and chemical properties at ambient, subcritical and supercritical conditions.

Table 1.12: Water physical and chemical properties at different conditions (Möller et al., 2011; Wu et al., 2008).

	Ambient temperature	**Subcritical water**	**Supercritical water**
Temperature (°C)	0-100	100-374	> 374
Vapor pressure (MPa)	0.003 (24 °C)	0.1 (100 °C) - 22.1 (374 °C)	> 22.1
Density, ρ (g cm^{-3})	Liquid 0,997 (25 °C)	Liquid 0.80 (250 °C, 5 MPa) 0.692 (330 °C, 30 MPa)	No phase separation Between gas-like and liquid-like densities, for instance, 0.17 (400 °C, 25 MPa)
Dynamic viscosity, η (cP)	0.89 (25 °C, 0.1 MPa)	0.11 (250 °C, 5 MPa)	Low 0.03 (400 °C, 25 MPa) 0.07 (400 °C, 50 MPa)
Heat capacity, Cp (kJ g^{1} K^{-1})	4.22 (25 °C)	4.86 (250 °C, 5 MPa)	13.0 (400 °C, 25 MPa) 6.8 (400 °C, 50 MPa)
Dielectric constant,ε	78.5 (25 °C, 0.1MPa)	27.1 (250 °C, 5 MPa) 18.2 (330 °C, 30MPa)	5.9 (400 °C, 25 MPa) 10.5 (400 °C, 50MPa)
Compressibility	No	Slightly increased, but still a liquid (at 370 °C)	Yes
Ion product, Kw	10^{-14} (increasing to 10^{-12} at 100 °C)	Increases from 10^{-12} (100 °C) to 10^{-11} (300 °C)	Strongly decreasing to below 10-20 (400 °C) and below 10-23 (550 °C);
Heat conductivity, λ (mW m^{-1} K^{-1})	608 (25 °C, 0.1 MPa)	620 (250 °C, 5 MPa)	160 (400 °C, 25 MPa) 438 (400 °C, 50 MPa)

1.7. Biomass hydrolysis using hot compressed water

Biomass fractionation is very important for a suitable and efficient use of cellulose, hemicellulose and lignin. HCW presents a high potential as a reaction media for biomass hydrolysis and fractionation due to its special physical-chemical characteristics, mainly a higher ionic product, comparing with the conventional methods normally used to hydrolyzed biomass (Hashaikeh et al., 2007).

Several studies demonstrated the potential of biomass fractionation using HCW by performing hydrolysis in a temperature range of 180-230 ºC, suitable for the recovery of fractions enriched in several components from each of the main biomass structures. At lower temperatures, occurs firstly the hydrolysis of vulnerable amorphous phases like hemicellulose and some fractions of lignin whereas cellulose is hydrolyzed at higher temperatures (Wu et al., 2008). *Ando et al.* studied the decomposition behavior of plant biomass in HCW and concluded that hemicellulose decomposition starts at 180 ºC while cellulose decomposition would not occur until the temperature is higher than 230 ºC (Ando et al., 2000). Although hydrolysis performance strongly depends on the nature of the biomass, a general behavior would be observed for the main types of available biomass which would include the collection of three different fractions. The first fraction collected would contain normally all the monosaccharides such as arabinose, glucose, fructose or disaccharides such as sucrose which are directly available in biomass and are extracted by HCW during the first period of hydrolysis processing. A second fraction includes hydrolyzed products from arabinoxylan, the main structure in hemicellulose, mainly arabinose, xylose and xylooligosaccharides. The third fraction includes glucose and cellooligosaccharides obtained from cellulose hydrolysis (Ando et al., 2000). This kind of mechanism would efficiently allow the recovery of different components avoiding their degradation into undesirable compounds.

Due to the complex hydrothermal reaction pathways, it is relatively difficult to optimize HCW hydrolysis processes of lignocellulosic materials. Regarding temperature manipulation, a two-step treatment using a tubular continuous flow reactor with a cooling system was proposed leading to a remarkable improvement in water-soluble compounds, mainly sugars, while preventing unwanted side degradation reactions (Wu et al., 2008). A two-stage HCW hydrolysis is a promising design to overcome the drawbacks associated with cellulose, hemicellulose, and lignin being hydrolyzed at different temperatures, thus improving biomass conversion and sugar recovery. Continuous flow type reactors promote higher sugar yields whereas hydrolysis products must be cooled rapidly to minimize degradation (Sakaki et al., 2002; Zhao et al., 2011). Considering willow dissolution as a model system for biomass conversion, the desirable approach consists on the recovery of hemicellulose-derived sugars at first operating at 180-200 ºC followed by an increase in temperature to over 230 ºC for the recovery of glucose from the cellulose structure (Hashaikeh et al., 2007) .

Mok et al. succeeded in the dissolution of most of hemicellulose and lignin contents from several woody and herbaceous biomass types using HCW at 190-230 ºC (Mok and Antal, 1992). *Ando et al.* studied the decomposition behavior of bamboo, chinquapin (hardwood) and Japan cedar (softwood) in hot compressed water achieving 95% of biomass extraction and fractionation after 60 minutes of reaction where free sugars, some lignin and most of hemicellulose were first solubilized in water operating at 180ºC. Cellulose started to decompose only at 230ºC. Actually, some of cellulose was dissolved at a relatively low temperature range of 215-230ºC which could be explained by a lower crystallinity of cellulose, having some amorphous regions which are can be hydrolyzed at milder conditions (Ando et al., 2000). Studies performed using several different species of biomass such as sugar bagasse (Karp et al., 2013), corn fiber (Zhou et al., 2014), and several woody and herbaceous biomass species (Mok and Antal, 1992) demonstrated that between 50-60% of biomass can be dissolved at temperatures of 180-240ºC, mainly the lignin and hemicellulose portions. However, a complete dissolution was not achieved as well as the main biomass components recovery varies regarding the residue in study. These studies demonstrate that depending on the nature of biomass, different HCW hydrolysis performances can be expected concluding that no standard conditions can be applied for all biomass types. However, it is possible to achieve high yields of biomass hydrolysis and fractionation using HCW but each biomass needs to be initially studied and treated as a single case.

The necessity to concentrate large amounts of biomass which are widely distributed, to transport it to the processing facility and high biomass variability, have hindered the development of a commercial process for HCW biomass hydrolysis (Brunner, 2009).

Biomass characteristics depend largely upon its origin which leads to significant variations in terms of chemical structure and composition. (Wu et al., 2008). HCW can also be used as a pretreatment followed by an enzymatic hydrolysis to overcome an incomplete lignocellulose hydrolysis. After hydrolysis, reaction by-products, mainly oligosaccharides sugars, are easily hydrolyzed into monosaccharides using enzymes. As an example, firstly rice bran was hydrolyzed using HCW at 200 ºC yielding only 5% of glucose and 35% of xylose. After a subsequent enzymatic treatment, it was possible to recover 70% of glucose and 70% of xylose relative to the maximum obtainable content (Schacht et al., 2008).

1.7.1. Cellulose hydrolysis

Cellulose can by hydrolyzed using HCW into cello-oligosaccahrides and glucose as well as into various degradation products such as pyruvaldehyde and 5-hydroxymethylfurfural (5-HMF) (Sasaki et al., 1998) or a final mixture of oil, char, gases and non-glucose aqueous products when operating at high temperatures (Minowa et al., 1998). Higher temperatures and low residence times result in high cellulose hydrolysis rates (Cantero et al., 2013). Cellulose hydrolysis using supercritical water at 400 ºC and 25 MPa with a residence time of 0,5 s was reported achieving 100% of conversion (Sasaki and Fang, 2000). Operating at lower temperatures, within the subcritical region, cellulose hydrolysis is also complete obtaining a high concentration of glucose and oligosaccharides, however, higher residence times are needed (Cantero et al., 2013).

Cellulose hydrolysis reaction pathway in subcritical and supercritical water was widely studied in the last years and includes several different reactions such as retro-aldol condensations, keto-enol tautomerism, and dehydrations (Ehara, K.; Saka, 2002; B M Kabyemela et al., 1997a; Bernard M Kabyemela et al., 1997). Nevertheless, these reaction rates could be controlled by manipulating temperature and pressure conditions in near- and supercritical water without using catalysts (Kabyemela et al., 1998; Sasaki and Fang, 2000; Wahyudiono et al., 2013)

Sasaki *et al.* investigated cellulose hydrolysis and its decomposition in a temperature range of 290-400 ºC (Sasaki et al., 1998) proposing later a mechanism and reaction kinetics of microcrystalline cellulose hydrolysis in subcritical and supercritical water at a pressure of 25MPa at the same temperature range (Sasaki et al., 2004). A simple scheme of cellulose hydrolysis reaction pathway is represented in figure 1.25.

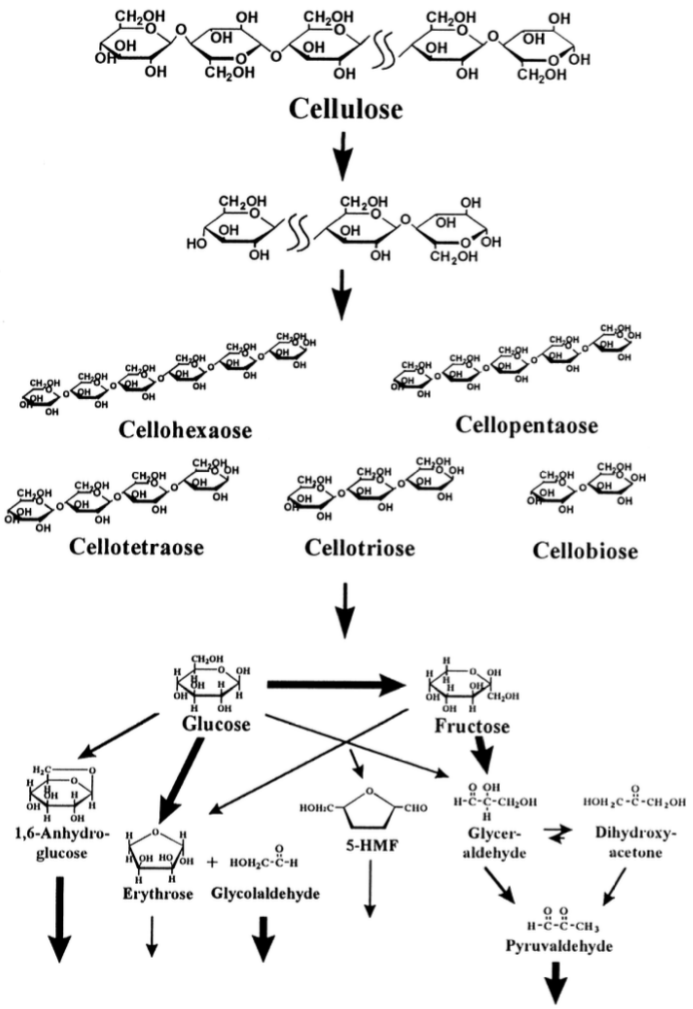


Figure 1.25: Cellulose hydrolysis main reaction pathway (Sasaki et al., 1998).

Cellulose is firstly hydrolyzed into complex oligomers to be further hydrolyzed into cellulose model compounds such us cellohexaose, cellopentaose, cellotetraose, cellotriose and cellobiose, the final pathway compound before glucose recovery (Figure 1.25). This first decomposition step into cellooligosaccharides is mainly done by two reaction pathways depending on the operating temperature: (Reaction 1) hydrolysis of the glycosidic bond via swelling or dissolution of cellulose or (Reaction 2) dehydration of the glucose reducing end molecule via thermal cleavage of the glycosidic bond in cellulose (Figure 1.26). Reaction 2 takes place as the temperature increase and at low pressures while Reaction 1 is predominant in high-density regions in near-critical and supercritical water (Matsumura et al., 2006a; Wu et al., 2008).

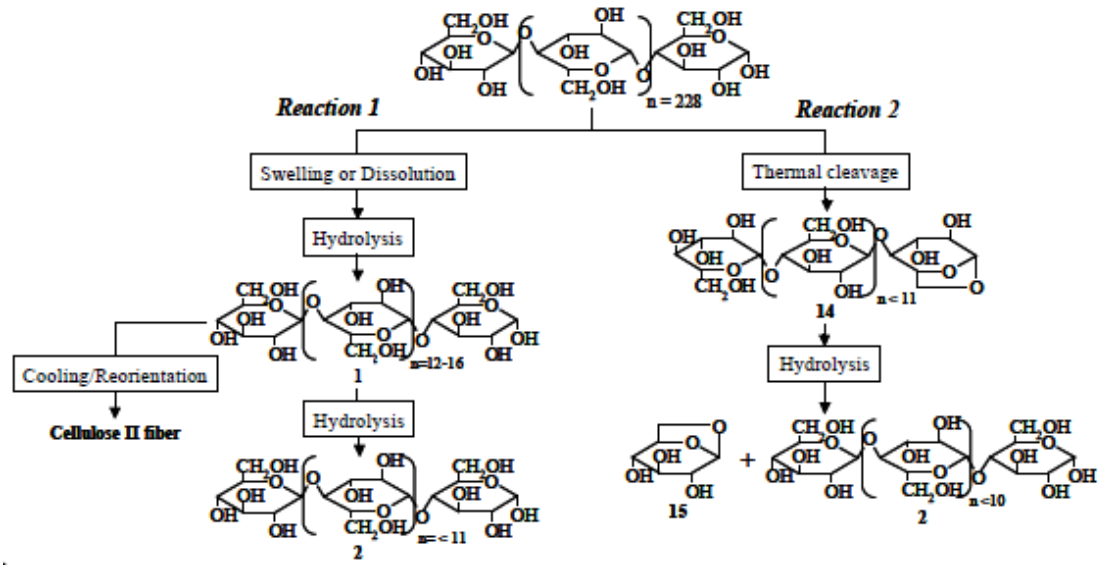


Figure 1.26: Reaction mechanism proposed for cellulose decomposition of microcrystalline cellulose in sub- and supercritical water. Addapted from (Matsumura et al., 2006a; Sasaki et al., 2003; Wu et al., 2008)**.**

At the last stage of cellulose hydrolysis, cellobiose units are obtained which are the precursors of the final glucose units. *Wu et al.*, *Sasaki et al* and *Kabyemela et al.* proposed the summarized reaction mechanism for cellobiose decomposition in HCW (Figure 1.27).

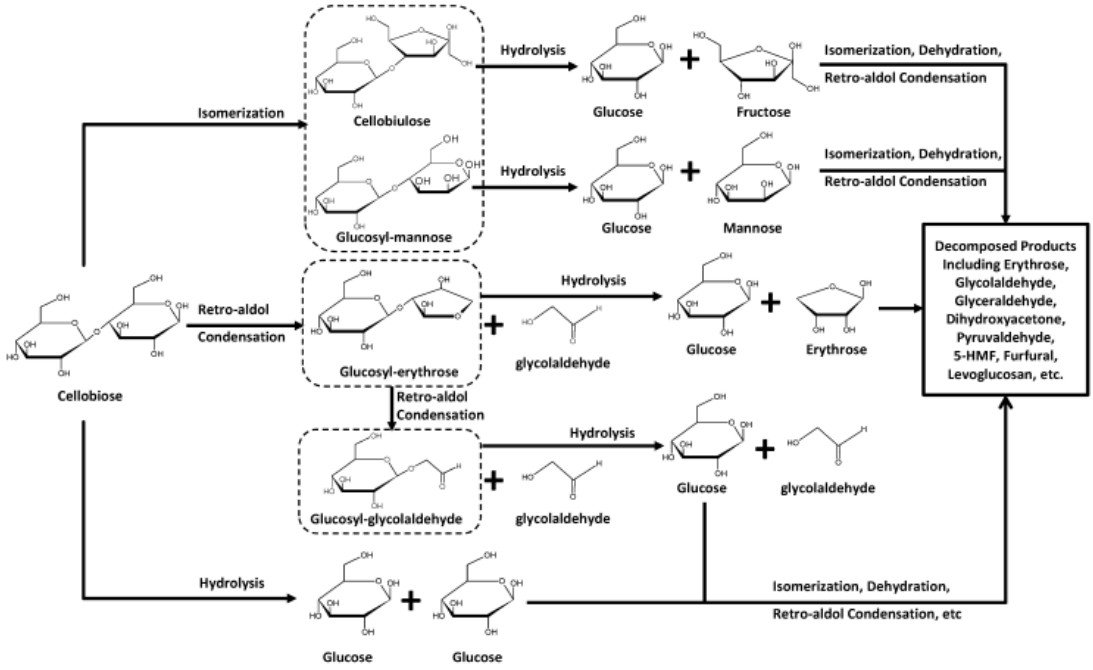


Figure 1.27: Summarized reaction pathways during cellobiose decomposition in HCW (Kabyemela et al., 1998; Sasaki et al., 2002a; Yu et al., 2013)

Cellobiose decomposition is composed of three main reaction pathways: (i) hydrolysis at the glycosidic bond yielding glucose; (ii) retro-aldol condensation reaction at the reducing end yielding glucosyl-erythrose and glycolaldehyde and (iii) isomerization reactions producing cellobiose isomers (Yu et al., 2013). *Sasaki et al.* have suggested that the contribution of each reaction path to the overall rate of cellobiose degradation can be controlled by temperature and pressure manipulation at near-critical and supercritical conditions whereas at lower water densities, retro-aldol condensation occurred predominantly and hydrolysis rates increased with an increase in the water bulk density (Sasaki et al., 2002a).

Wu et al. clearly showed that isomerization reactions and retro-aldol condensations are the major primary reactions during cellobiose decomposition in HCW with the formation of two cellobiose isomers, cellobiulose and glucosyl-mannose and glucosyl-erythrose. The formation of glucosyl-glycolaldehyde by glucosyl-erythrose retrol-aldol condensation is a minor reaction pathway which occurs only at cellobiose conversions higher than 25%. The production of glucose via cellobiose hydrolysis only accounts for 10-20% of the total primary products of cellobiose decomposition reactions at the early stage (Yu et al., 2013). The primary products from isomerization and retro-aldol condensation may be further decomposed into glucose, fructose, mannose and erythrose. Other secondary reactions such as dehydration could also occur and further decompose sugar products into degradation products, e.g., dihydroxyacetone, pyruvaldehyde, 5-HMF, furfural, among others (Kabyemela et al., 1998). These degradation reactions pathways will be further discussed in section 1.7.4. of this chapter.

Sasaki et al. (figure 1.28) proposed a reaction mechanism for microcrystalline cellulose hydrolysis at subcritical and supercritical water conditions.

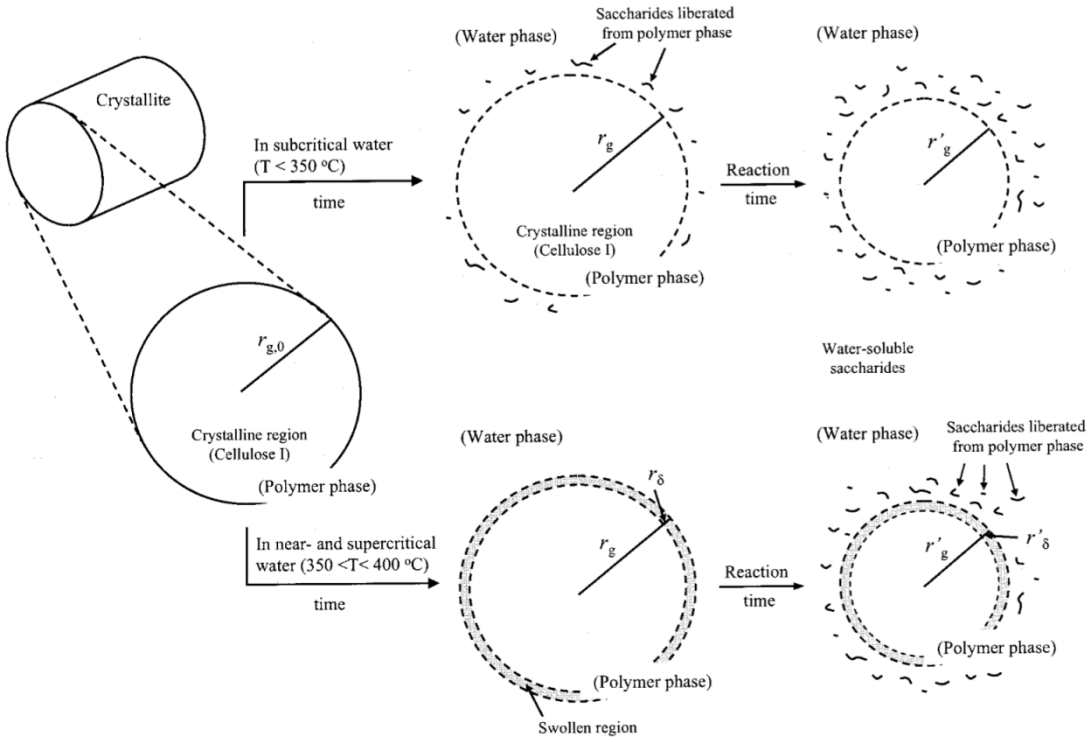


Figure 1.28: Reaction mechanism proposed for the conversion of microcrystalline cellulose at subcritical and supercritical conditions, r_g- internal radius. (Sasaki et al., 2004).

In subcritical water, the crystallite is hydrolyzed at the surface region without swelling or dissolving as presented in the upper part of figure 1.28 and consequently the overall conversion rate of microcrystalline cellulose is slower. On the other hand, at near and supercritical conditions, the crystallite can swell or dissolve around the surface and form amorphous-like cellulose which is easily hydrolyzed to lower DP celluloses and cellooligosaccharides. Some of those amorphous molecules can be released for the water phase by cleavage of their hydrogen bonds and be further hydrolyzed to water-soluble saccharides or even re-crystalized as water-insoluble cellulose II. Moreover, the amorphous-like zone that remains in the polymer phase can be hydrolyzed to water-soluble saccharides, swell further or dissolve into the water phase (Cantero et al., 2013). This mechanism suggests that at subcritical conditions, heterogeneous cellulose hydrolysis occurs, while at near and supercritical conditions, cellulose hydrolysis occurs in a homogenous phase due to a complete dissolution of cellulose (Sasaki and Fang, 2000). Adschiri's group has performed direct observation of cellulose decomposition in supercritical water, using a diamond anvil cell (DAC) and observed that cellulose could be dissolved in water at the supercritical region (Figure 1.29) (Sasaki et al., 1998)(Sasaki and Fang, 2000)(Arai and Adschiri, 1999).

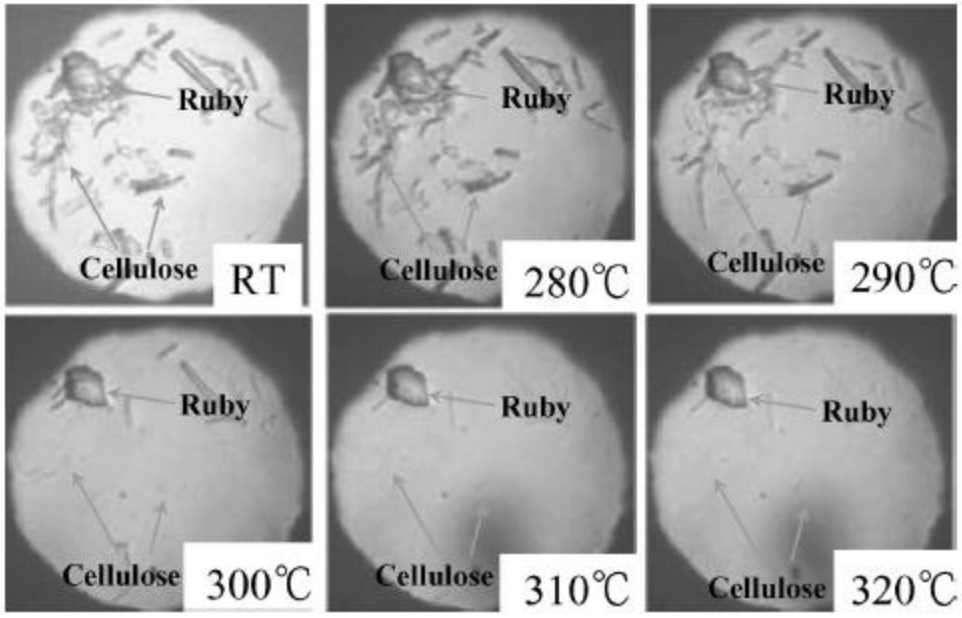


Figure 1.29: Cellule-water system phase behavior observation (Matsumura et al., 2006a)**.**

1.7.2.Hemicellulose hydrolysis

In contrast to cellulose, hemicellulose is a heteropolymer consisting of repeating pentoses units (xylose and arabinose) and hexoses units (glucose, galactose and mannose) branched together into short polymers in an amorphous and hydrophilic structure (Pinkowska et al., 2011). The two major classes of hemicelluloses are xylans and glucomannans (Fan et al., 2009).

Currently, biomass hydrolysis mechanisms models are mostly built considering cellulose hydrolysis behavior (Wu et al., 2008).

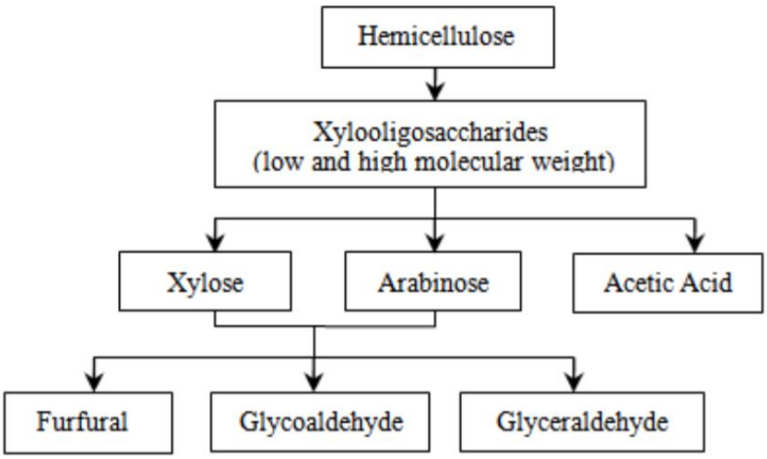


Figure 1.30: Main reaction pathways of hemicellulose hydrolysis (Cardenas-toro et al., 2014)**.**

In general, hemicellulose is hydrolyzed into high and low molecular weights oligomers, xylose, arabinose and degradation compounds such as furfural (Figure 1.30). It was proven that most of the xylose released from xylan fraction of hemicellulose by HCW hydrolysis is in its oligomeric form where the xylooligomer is then decomposed in xylose (Jacobsen and Wyman, 2002). Longer residence times could increase xylose monomer recovery though at the expense of decreasing the total yield due to the increase of further xylose degradation reactions into furfural and other degradation products.

Previous studies reported a recovery of hemicellulose as solubized compounds by HCW hydrolysis between 30-86%, for different types of biomass (Cardenas-toro et al., 2014; Pinkowska et al., 2011; Sabiha Hanim et al., 2012). Six woody and four herbaceous biomass samples were treated with HCW at 200-300ºC for 0-15min leading to 40-60% of the total mass solubilized. In all cases, 100% of hemicellulose was solubilized from which an average of 90% was recovered in monomeric sugars form (Mok and Antal, 1992).

Hemicellulose sugar distribution is highly dependent on the type of biomass and its origin. Hemicellulose hydrolysis produces mainly xylooligosaccharides with different structures and compositions (Fang, Z.; Xu, 2014)**.** *Nabarlatz et al.* developed a study related with hemicellulose hydrolysis of several different lignocellulosic materials concluding that xylooligomers formed were mainly 4-O-methylglucuronoxylan with different degrees of acetyl groups substitution (Nabarlatz et al., 2007). Hydrothermal treatment of hemicellulose at temperatures of approximately 200 ºC originates a slightly acidic medium due to the partial cleavage of the hemicellulose acetyl groups yielding high molecular weight xylooligosaccharides with a significant content of acetyl and uronic groups which confer them a high solubility in water (Cardenas-toro et al., 2014). The total dissolution of these groups promotes an increase of hydrogen ions in solution which leads to hydrolytic degradation of the polymer chains into oligosaccharides (xylo- and arabinooligosaccharides), monosaccharides (xylose, arabinose and glucose), furfural and non-saccharide degradation compounds (Garrote et al., 2004). Additionally, arabinosyl and acetyl groups were found to be less resistant to hydrolytic cleavage than uronic groups (Branson et al., 2010).

A more detailed study about hemicellulose degradation reaction mechanism can be performed with commercially available xylan, which plays the role of a model substance for hemicellulose. Its main components are xylopyranose compounds organized in a polymer chain and linked by β-(1,4)-glycosidic bonds and depending on the origin, xylan backbone may contain side groups as arabinofuranose, acetyl, 4-O- methylglucuronic groups, among others (Wyman et al., 2005) *Pinkowska et al.* proposed a hydrothermal decomposition pathway for xylan in subcritical water at a temperature range of 180-300ºC (Pinkowska et al., 2011).

From figure 1.31, it can be observed that xylan hydrothermolysis starts with (1) its depolymerization and acetyl groups cleavage leading to (2) xylose, (3) arabinose and (4) acetic acid production. At the second reaction stage, pentoses are dehydrated and subjected to retro-aldol condensation reactions resulting in the production of (5) furfurals and (6) 5-HMF. Further consecutive hydration, dehydration and tautomerization reactions lead to the formation of (7) glycolaldehyde and (8) glyceraldehyde from retro-aldol condensation of xylose and arabinose, to (9) formic acid, (10) dihydroxyacetone from glyceraldehyde tautomerization reaction and (11) pyruvaldehede from glyceraldehede dehydration reaction. Regrouping and dehydration of pyruvaldehyde might result in the production of (12) lactic acid.

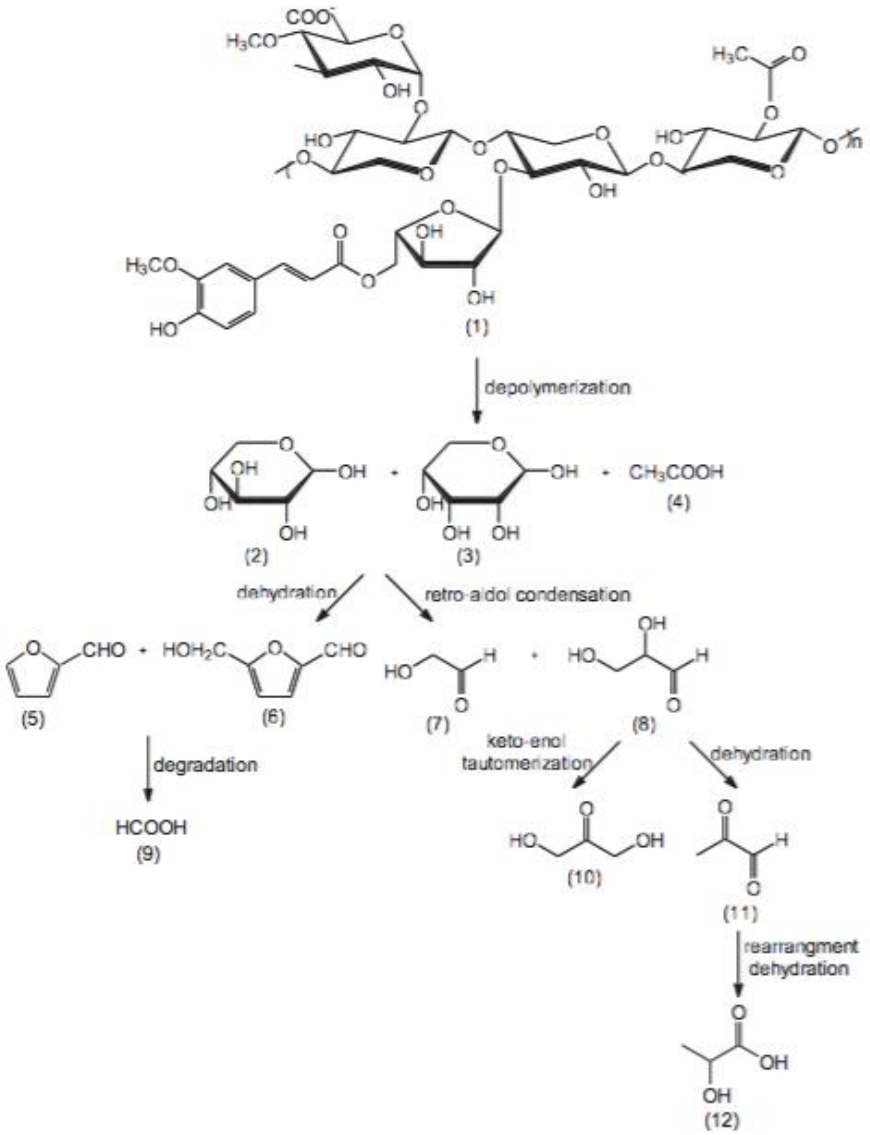


Figure 1.31: Hydrothermal xylan decomposition pathway in sub-critical water (Pinkowska et al., 2011).

1.7.3.Lignin hydrolysis

Lignin is a potentially renewable source of natural aromatic chemicals which has driven the increasing interest in research towards its valorization for chemical and liquid fuel production (Toledano et al., 2011)**.**

Lignin decomposes more slowly than cellulose and hemicellulose components and over a broader range of temperature (200 – 500 ºC) since several functional groups containing oxygen from its structure have different temperature stabilities with their cleavage occurring at different temperatures (Caballero et al., 1997). The lignin pyrolysis process starts at relatively low temperatures of 200 – 275 ºC and the main process occurs around 400 ºC with the formation of aromatic hydrocarbons, phenolics, hydroxyphenolics and guaiacyl/syringyl-type compounds containing hydroxyl groups (Rodrigues et al., 2001). The major pyrolysis products of lignin include gaseous hydrocarbons such as carbon monoxide and carbon dioxide, volatile liquids (methanol, acetone, and acetaldehyde), monolignols, monophenols (phenol, guaiacol, syringol, and catechol), and other polysubstituted phenols (Pandey and Kim, 2011) .

Different thermochemical methods for lignin depolymerizing into high-value aromatic compounds have been studied, including fast pyrolysis, wet oxidation and hydrothermal processing. In general, the degradation of lignin into its constituent compounds proceeds by depolymerization and deoxygenation reactions (Wahyudiono et al., 2011). However, during these reactions, condensation and repolymerization of intermediate products also takes place leading to the formation of char resulting in poor aromatic chemicals yields (Onwudili, J.A.; Williams, 2013). Several processes have been investigated in order to minimize char formation including the use of ionic liquids (Cox and Ekerdt, 2012) and capping agents such as boric acid (Roberts et al., 2011) and phenol (Toledano et al., 2014) as well as the use of organic solvents as depolymerization media (Song et al., 2013). Ionic liquids and organic solvents used as the reaction medium for lignin depolymerization has led to high conversion rates with significant aromatic chemicals yields. However, several issues are raised regarding solvent recovery as well as the use of fossil-based solvents, such as organic solvents, which have a strong impact on the process sustainability (Onwudili, J.A.; Williams, 2013).

Several studies have been reported using hot compressed water for lignin depolymerization with the use of catalysts addition such as acid, base and metal catalysts and under supercritical conditions (Zakzeski et al., 2012)(Jin et al., 2006)(Wahyudiono et al., 2011). Lignin depolymerization using hot compressed water is becoming more attractive to researchers than supercritical water due to the lower temperatures and pressures applied which may reduce costs. Most major ether bonds in lignin are between one propyl side chain of a hydroxylphenylpropane unit and the hydroxyl group linked to the benzene ring of a different hydroxylphenylpropane unit. The breakage of ether bonds and carbon–carbon bonds is an important factor in the degradation of lignin (Wahyudiono et al., 2008). Lignin

structure hydrolysis takes place at ether and ester bonds cleaving them by the addition of one molecule of water for every broken linkage (Figure 1.32) (Bobleter, 1994). Because lignin depolymerization is promoted by higher ionic product of water at subcritical conditions, HCW technology may result in an alternative fractionation of biomass components.

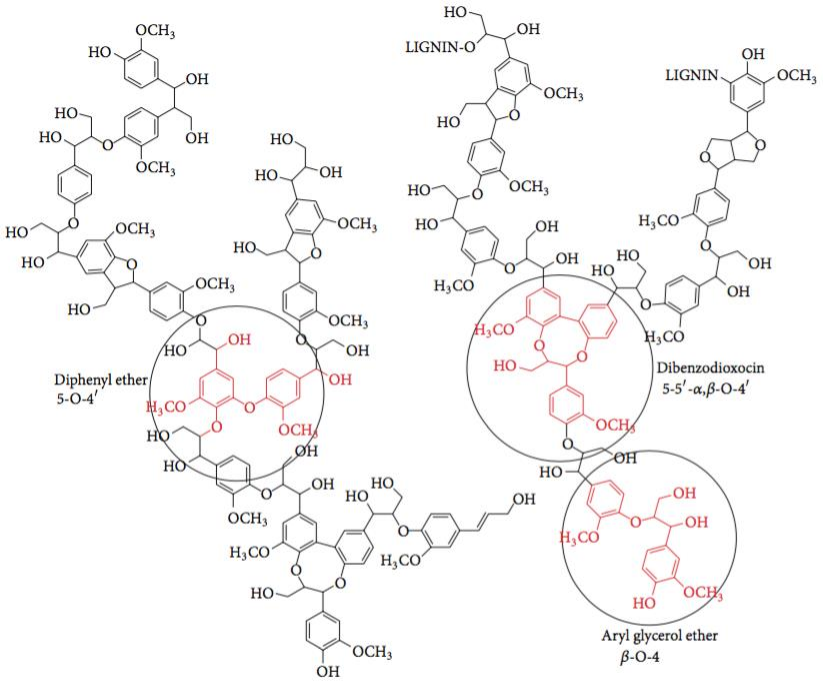


Figure 1.32: Common lignin chemical structure with ether linkages between hydrophenylpropane units (Wang et al., 2013).

Machmudah et al. reported a lignin recovery of nearly 85% from Japanese rice straw using hot compressed water in a continuous flow reactor at 170-200 ºC at 4.0 MPa, with a water flow rate of 4.67 ml/min. Under these conditions, thermal softening of the Japanese rice straw occurred allowing the removal of the lignin (Machmudah et al., 2015).

Saisu et al. and *Okuda et al.* proposed a simple mechanism for lignin depolymerization at sub-critical and supercritical water conditions (Saisu et al., 2003). Lignin first decomposes by hydrolysis and dealkylation reactions where the cleavage of oxygen functional groups originates formaldehyde, syringols, guaiacols, catechols and phenols. A cross-linking between formaldehyde and low-molecular fragments from lignin depolymerization occurs leading to the formation of high molecular weight compounds due to a rearrangement of the backbone main structure. The cleavage of the aryl–ether linkages results in the formation of highly reactive and unstable free radicals that may further react through rearrangement, electron abstraction or radical–radical interactions, to form more stable products such as char and volatile products (Brebu and Vasile, 2010). Additionally, the presence of

phenol as a solvent prevented cross-linking capturing the reactive fragments during lignin depolymerization and decreasing the formation of insoluble compounds (Saisu et al., 2003). *Okuda et al.* reported that lignin decomposition is completed in 6 min to form species of around 1000 g/mol average molecular weight and other low molecular weight compounds with 100 to 300 g/mol using a mixture of water-phenol at 300 ºC. By increasing reaction time, the amount of the higher molecular weight compounds decreased at the same time that lower molecular weight compounds content increase without char formation (Okuda et al., 2004).

Lignin depolymerization mechanism is extremely complex and highly influenced by several factors such as lignin nature, reaction temperature and heating rate (Brebu and Vasile, 2010). The aromatic structure and random lignin inter-unit linkages make it chemically and physically difficult to predict its behavior when lignin is exposed to elevated temperatures since changes can occur in its chemical structure that affect its performance during hydrolysis reactions (Palmer, 1983; Wahyudiono et al., 2013). Further complexity arises when the lignin has been generated as a by-product from an industrial application since different chemical processes yield different types of lignin wastes (Lin et al., 2015) .

1.7.4. Degradation reactions of monosaccharides

As already mentioned, monosaccharides derived from biomass hydrolysis can be easily decomposed into several products. Hot compressed water hydrolysis reactions are often accompanied by thermal reactions such as retro-aldol and dehydration reactions. The higher the temperature, the higher will be the extension of those thermal reactions (Brunner, 2009). Additionally, since the decomposition of hemicellulose starts to occur at lower temperatures (~180ºC) than that of cellulose (~230ºC), the degradation of monosaccharides derived from hemicellulose hydrolysis would be unavoidable if hemicellulose and cellulose are hydrolyzed together (Khajavi et al., 2005; Matsumura et al., 2006b; Oomori et al., 2004).

Glucose, fructose and xylose decomposition mechanisms have been widely studied with or without catalyst (Bobleter, 1994; B M Kabyemela et al., 1997a, 1997b; Kabyemela et al., 1999). The degradation of monosaccharides includes several chemical reactions such as isomerization, tautomerization, hydration, dehydration and cyclization (Antal, M.J.;Leesomboon, T.; Mok, 1990; Fang, Smith, Kozinski, Minowa, & Arai, 2011; Huber, Iborra, & Corma, 2006). In the subcritical region, glucose decomposition is mainly conducted by dehydration reactions whereas at higher temperatures, mainly in supercritical region, retro-aldol condensations are predominant (Wu et al., 2008). Moreover, at subcritical conditions, glucose degradation rate is much faster that its formation from cellobiose hydrolysis. Near the critical point, cellobiose degradation rate increases

approximately one order of magnitude and thereby glucose formation rate exceeds its degradation rate (Fang, Z.; Xu, 2014).

The main identified and reported reaction pathways for glucose and fructose degradation reactions in subcritical water are described in figure 1.33.

It is important to enhance that in equilibrated aqueous solutions, monosaccharides are present as different species. For instance, at room temperature, D-glucose presents 3 distinct forms: β-pyranoidic, α-pyranoidic and β-furanoidic as well as in the open chain form (Collins, 2006a); D-fructose 4 distinct forms: β-fructopyranose, α-fructopyranose, β-fructofuranose and α-fructofuranose as well as in the open chain form (Collins, 2006b). Furthermore, glucose isomerization into fructose, known as Lobry de Bruyn-Alberda van Ekenstein transformation (LBET), is via the open chain form and proceeds preferably at high pH. However, in subcritical water, the increase on pK_w together with the increase of temperature, triggers the conversion of glucose into fructose at lower pH values (Möller et al., 2011). Glucose processed during subcritical water hydrolysis yields measurable amounts of fructose whereas a negligible amount of glucose was obtained from fructose, which may be attributed to a slower reaction kinetics towards direction of glucose formation (Aida et al., 2007).

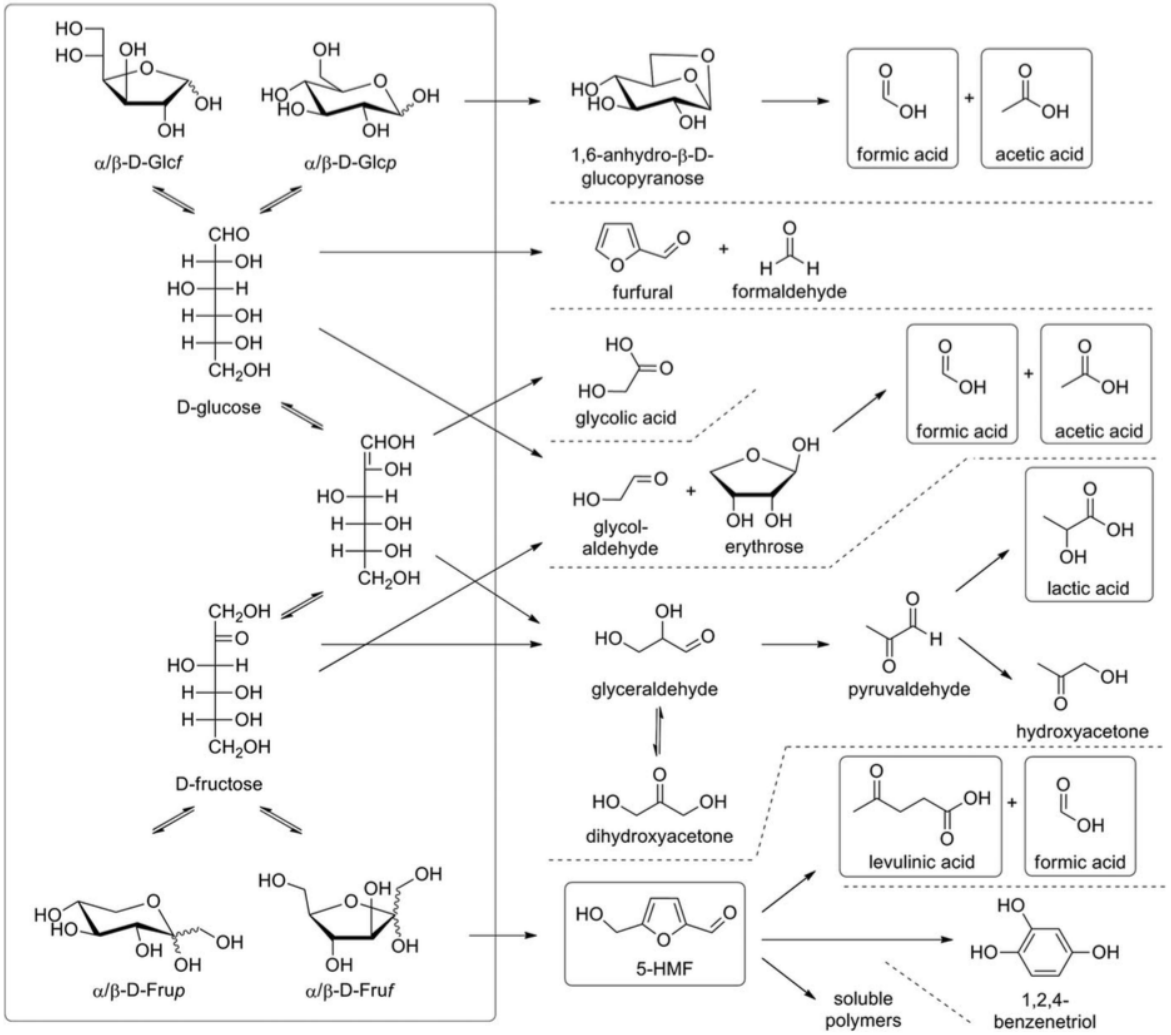


Figure 1.33: Main reaction pathways of glucose and fructose in subcritical water. (Möller et al., 2011)

The primary reactions for glucose decomposition are: (i) glucose isomerization into fructose via keto-enol tautomerization; (ii) glucose dehydration into 1,6-anhydroglucose; (iii) glucose decomposition into aldehyde and ketone via retro-aldol condensation and (iv) dehydration of the tautomerization intermediate and 5-HMF production from fructose (Wu et al., 2008). 5-HMF is also formed directly from glucose and fructose and since it is a relatively stable molecule, high yields are obtained with long residence times (Sasaki et al., 1998). Produced fructose can also decompose to erythrose and glyceraldehyde or glyceraldehyde and dihydroxyacetone. Furthermore, glyceraldehyde can convert into dihydroxyacetone and both glyceraldehyde and dihydroxyacetone dehydrate into pyruvaldehyde. Pyruvaldehyde, erythrose and glycolaldehyde can be further decomposed to smaller molecules which are mainly acids, aldehydes and alcohols of 1-3 carbons chains. At higher temperature, oil, char and gases can also be produced as the final stage of the degradation reactions of glucose (Jong, W.; Ommen, 2014). At lower temperatures, methane formation is promoted while at higher temperatures, mainly in supercritical region, hydrogen formation is preferential (Brunner, 2009).

Summarizing, degradation products formed from the subcritical water processing of glucose and fructose can be distinguished, according with the type of degradation reaction:

- Fragmentation products such as glyceraldehyde, dihydroxyacetone, pyruvaldehyde, erythrose, glycolaldehyde and hydroxyacetone obtained from retro-aldol condensation reactions from glucose and fructose (Sasaki et al., 2002b). Acetic acid can be formed by the fragmentation of 1,6-anhydro-β-D-glucopyranose or by decomposition of erythrose (Kabyemela et al., 1999).

- Dehydration products such as 6-anhydroglucose and furfural which is derived from a hexose fragmentation followed by a dehydration and 1,2,4-benzetriol (5-HMF secondary dehydration product) (Ehara and Saka, 2005; Luijkx, G.C.A; Rantwijk, F.;Bekkum, 1993). 5-HMF is also easily decomposed into levulinic and formic acids (Aida et al., 2007). 5-HMF is the major degradation product from hexoses and furfural is the major degradation product from pentoses. (Fang, Z.; Xu, 2014) Higher yields of 5-HMF are formed deriving from d-fructose while higher yields of furfural were originated by d-glucose dehydration (Brunner, 2009). Formic acid can also be formed through glucose dehydration or from erythrose decomposition (Peterson et al., 2008).

- Condensation products mainly soluble and insoluble polymers and carbonized products. (Titirici et al., 2007).

- Gaseous molecules derived from small molecules decomposition which includes CO_2, H_2 and CO (Chheda et al., 2007).

In addition to all these final products obtained from fructose and glucose degradation reactions, other compounds can also be obtained in small amounts or as intermediate compounds such as low molecular weight carboxylic and dicarboxylic acids, e.g., propionic, acrylic, maleic, maloic, succinic acids and ketones, e.g., 2-butanone and 2,5-hexandione (Möller et al., 2011); (Kabyemela et al., 1999).

Under acidic conditions, dehydration reactions of glucose are favored and the main degradation compounds are 5-HMF and levulinic acid whereas under alkaline conditions, retro-aldol condensation reactions are favored and carboxylic acids such as lactic and acetic acids are predominant (Wu et al., 2008). Under neutral conditions, both acidic and basic conversion products are formed due to promoted self-dissociation of water at high temperatures (Yin and Tan, 2012). General pathways for glucose and fructose degradation reactions according acidic, alkaline and neutral conditions are schematically represented in figure 1.34. Another aspect to enhance is that some organic acids can act

as homogenous catalysts promoting monosaccharides decomposition. For instance, fructose degradation is increased in the presence of formic and acetic acid probably due to the increase of proton concentration and the resulting acid hydrolysis (Möller et al., 2011).

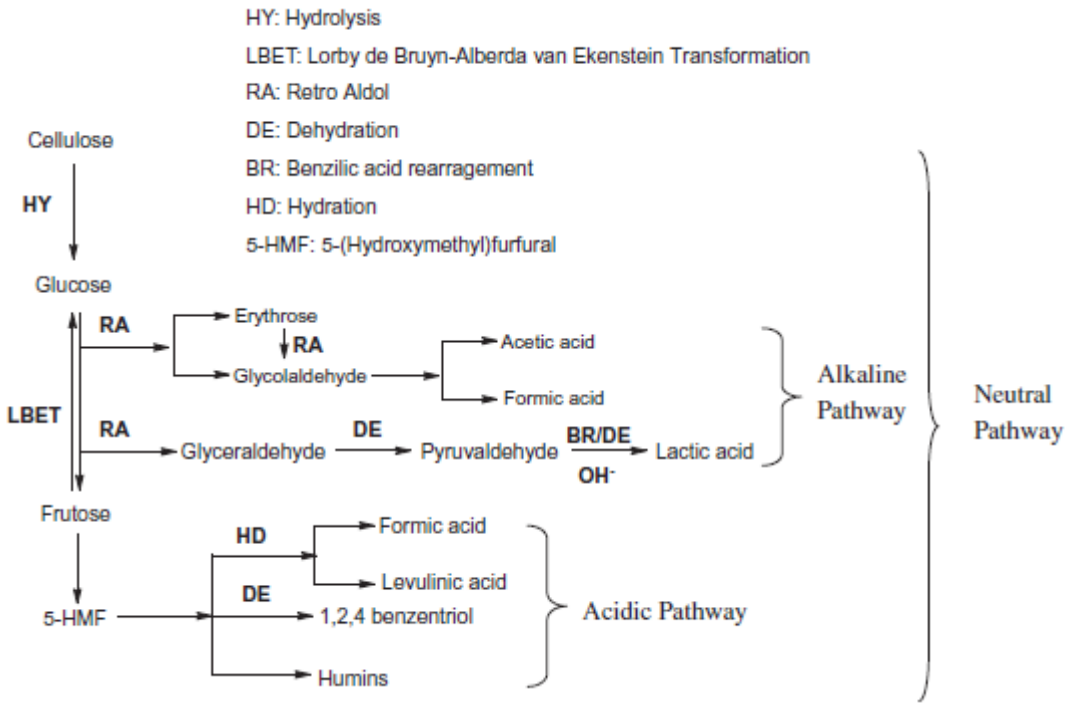


Figure 1.34: General pathways for cellulose hydrothermal decomposition according to acidic, alkaline or neutral conditions (Yin and Tan, 2012).

Sasaki et al. presented in figure 1.35 the reaction mechanisms for xylose degradation, a monomer of the xylan component from hemicellulose structure (Sasaki, M.;Hayakawa, T.; Arai, K.; Adschiri, 2003).

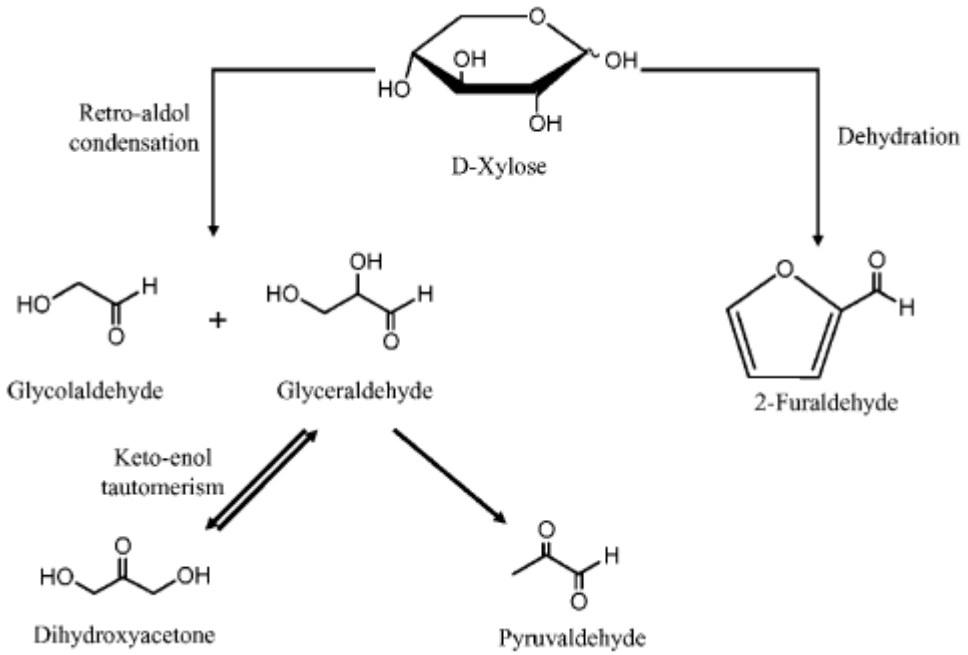


Figure 1.35: Main reaction pathway for D-xylose degradation (Sasaki, M.;Hayakawa, T.; Arai, K.; Adschiri, 2003)**.**

The kinetic study of this reaction mechanism demonstrated the consistency of the retro-aldol and dehydration reactions for the overall degradation mechanisms as in the case of glucose in sub- and supercritical water. Some other derived sugars from hemicellulose such as glucose, mannose, galactose and arabinose can be obtained from hemicellulose hydrolysis and were studied by *Srokol et al.* in order to evaluate hydrolysis and degradation behaviors differences between five and six-carbon sugars (Srokol et al., 2004). No significant differences were found in the products obtained from six-carbon degradation although the amounts of the various compounds and their respective formation rates strongly depend upon the nature of the starting sugars. The decomposition products of arabinose were mainly glycolaldehyde and 2-furaldehyde, which are also the main degradation products from xylose (Figure 1.35). It was concluded that the decomposition mechanisms of hemicellulose derived six- and five- carbon sugars are similar in HCW (Srokol et al., 2004).

1.8. Added value products extraction using hot compressed water extraction

Valuable compounds, such as phenolic compounds, can be extracted from waste materials applying conventional extraction methods. Solid-liquid extraction using organic solvents is the most common technique for obtaining phenolic-rich extracts, mainly, maceration and Soxhlet extraction (Tahir, 2015). Extraction efficiencies depend largely on solvent polarity as well as the extraction temperature. The most used solvents are ethanol, methanol, acetone, water and also acidified solutions (Azmir et al., 2013). The major drawbacks of these conventional methods include long extraction times, the use

of large volumes of organic solvents, poor extraction selectivity and the generation of large amounts of organic wastes (Eikani et al., 2007).

In order to establish environmental friendly and benign process methods, researchers have made major efforts to introduce new methods and techniques to overcome challenges faced during extraction procedures. New technologies have been developed during the last decade such as supercritical fluid extraction, mainly using CO_2 (Fornari et al., 2012), accelerated solvent extraction by using the conventional solvents but applying high temperatures and pressures, in order to increase the extraction efficiency due to deeper penetration of the solvent in the sample matrix pores (Barros et al., 2013); enzymatic hydrolysis where a pretreatment is applied in order to improve water extractions efficiency due to an increase on phenolic compounds accessibility (Ribeiro et al., 2013) (Chua, 2013); microwave-assisted extraction and ultrasound-assisted extraction, which consist in using microwaves or ultrasounds to cause cell disruption, increasing mass transfer and facilitating solvent access (Zeng et al., 2010) (Yang et al., 2011) and pressurized liquid extraction such as hot compressed water extraction (Teo et al., 2010).

Several parameters affect HCW extraction efficiency such as, biomass origin, temperature, pressure, time and solid-to-water ratio (Ndlela et al., 2012), samples particle size, pH and flow rate. (Khajenoori et al., 2013).. At lower temperatures, ionic and polar species will be preferably extracted while at higher temperatures, mainly when approaching the critical temperature of water, nonpolar substances will be readily dissolved and extracted (Brunner, 2009). As temperature increases, water viscosity and surface tension decreases, increasing mass transference rate and, consequently, extractable compounds solubility (Asl, A.H.; Khajenoori, 2013). Temperature is a crucial parameter to take into account when using HCW for extraction processes. (Wiboonsirikul and Adachi, 2008) An optimal balance between degradation reactions and extraction has to be taken into consideration. Although hydrolysis and extraction yield increases with temperature, some thermally sensitive compounds may be degraded after being released from the sample matrix (Wiboonsirikul and Adachi, 2008). It was reported by *Alvarez et al.* that increasing temperature from 100 to 220ºC, increased the total amount of phenolic compounds extracted from potato peel. At temperatures higher than 220ºC, a decrease in the extraction yield was observed. The authors attribute this decrease in the extraction yield due to a possible product degradation accompanied by a decrease on pH (Alvarez and Saldaña, 2013).

Time also influences HCW extraction. Long extraction time can increase the yield of material recovery as it was reported by He *et al.* where 1510 mg of total phenolic compounds/ 100 g were extracted from pomegranate seeds within 30 minutes and 1890 mg/100 g within 120 min (He et al.,

2012). However, the increase in the amount of extract recovered after 30 min of extraction time probably does not sustain the increase in process costs to maintain the operation for another 90 min.

Recently, several studies using HCW treatment performed to extract phenolic compounds from fruit peels, seeds, food matrices, among others, have demonstrated that HCW technology is a promising and highly effective method to replace conventional methods (Tahir, 2015). *Monrad et al.* designed and optimized a grape pomace continuous flow extraction system using only water achieving 96% of anthocyanins and 84% of procyanidins extraction yields when comparing with solvent extraction with methanol- or acetone-based conventional solvents. (Monrad et al., 2012). *Singh and Saldaña*, performed the phenolic compounds extraction from potato peel using also subcritical water and they found that, a total of 81.83 mg/g of phenolic compounds were recovered at 180 °C within 30 minutes of extraction time as compared to 46.59 mg/g in 3 hours using methanol (Singh and Saldana, 2011). In a similar study, *Tunchaiyaphum et al.* made a direct comparison for phenolic compounds extraction methods from mango peel by using HCW and Soxhlet method with ethanol 95% from mango peel. The highest phenolic content of 50.25 mg gallic acid equivalents (GAE)/g dried weight was obtained by using HCW at 180°C with a extraction time of 90 minutes while using soxhlet extraction techniques, the extraction time was higher than 120 minutes (Tunchaiyaphum et al., 2013). Therefore, researchers suggested the viability of replacing the conventional methods using organic solvents by HCW extraction as a green alternative technology for phenolic compounds extraction from agricultural wastes (Tahir, 2015).

1.9. Oleaginous yeast

Currently, single cell oils, which are lipids produced by oleaginous unicellular microorganisms, attract much attention of researchers. Traditionally, microorganisms such as microalgae, bacteria, fungi and yeast, which under specific cultivation conditions accumulate lipids up to 20% or higher of their dry weight are called oleaginous microorganisms (Beopoulos et al., 2009). Oleaginous microorganisms represent an attractive alternative to higher plants to convert waste materials, mainly agroindustrial residues, into valuable chemicals such as lipids, since their production does not require agricultural land avoiding displacement of food production (Santamauro et al., 2014). Recently, yeasts have gained a great biotechnological interest with their ability to generate several compounds with numerous important applications in food, cosmetics and pharmaceutical industries.

Oleaginous yeasts can, on average, accumulate lipids to higher than 40% of their dry weight using several different carbon sources. Under different nutrient-deficient conditions , they may accumulate intracellularly storage lipids to levels exceeding 70% of their biomass (Beopoulos and Nicaud, 2012). The most well studied oleaginous yeasts include species of *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, *Yarrowia*, *Lypomyce*s among others. Nevertheless, lipid contents, profiles and

accumulation potential can vary among different species and also within closely related species and among strains of the same species. For instance, in *Rhodotorula* species, lipid contents can differ significantly since *Rhodotorula glutinis* can accumulate lipids to levels higher than 70% whereas *Rhodotorula graminis* accumulate to levels of around 36% (Beopoulos and Nicaud, 2012), while fatty acid composition remains similar. *Yarrowia lipolytica* is known for its low capacity to accumulate lipids, comparing with other oleaginous yeasts, but it represents the only yeasts able to accumulate more than 50% of linoleic acid, in proportion, of the total fatty acids (Beopoulos et al., 2009).

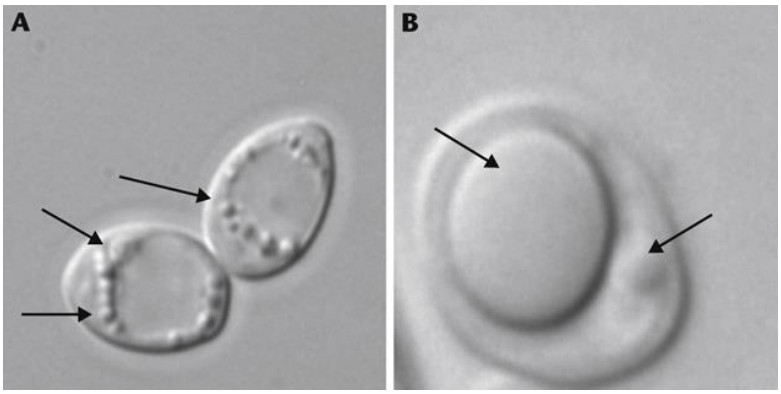


Figure 1.36: Optical microscope picture of *Yarrowia lipolytica.* Arrows identify lipid droplets. (A) Wild type growing on glucose-based media. (B) Over lipids accumulation corresponding to *c.a.* 60% of the yeast cell dry weight in a mutant strain growing on oleic acid medium. (Beopoulos et al., 2008)

In general, basidiomycetous yeast species are able to utilize a broader array of carbon sources than ascomycetous yeast species such as *S. cerevisiae* and *Y. lipolytica* and are more likely to be able to grow without supplemented vitamins (Kurtzman, C.; Fell, J.; Boekhout, 2011).

In oleaginous yeast, lipids are found mainly in the form of neutral lipids, triacylglycerols (TAGs), diacylglycerols (DAGs), monoglycerols, sterylesters, free sterols, free fatty acids (FFA) and polar fractions with glycolipids and phospholipids (Dey and Maiti, 2013). FFAs may constitute more than 30% of the total accumulated lipids (Tsigie et al., 2011). Of the neutral lipids, 80–90% are TAG (Radulovic et al., 2013). Triacylglycerols and steryl esters (SE) are the major cellular lipids synthesized from sterol, glycerol-3-phosphate, and acyl-CoA. Since these lipids are insoluble in the cytoplasm, they gather to form a hydrophobic core of the storage compartment called oleosome or lipid body (LB) (Han et al., 2013). LB consists of a core formed by neutral lipids surrounded by a phospholipid-protein monolayer (Figure 1.37).

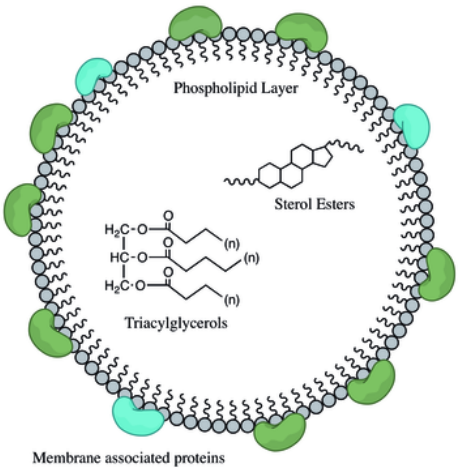


Figure 1.37: Schematic representation of a lipid body (Martin, S.; Parton, 2006).

Several researchers have been focusing their studies on reducing the cost and improving the productivity of single cell oil production. Screening for optimal oleaginous microorganisms capable of producing higher amount of lipids with structure and composition similar to that of high-value fats and able to produce them at large-scale became a key mission in the field of single cell oil production. In parallel, it is extremely necessary to find efficient and lower-cost feedstock carbon substrates alternatives. Several oleaginous microorganisms can present remarkable growth, and production of single cell oils, on wastes and by-products of the agroindustrial sector, thus, valorization of these residues together with production of potentially high-added value lipids could increase the viability of the process being simultaneously beneficial for the environment (Papanikolaou, 2012). Because yeast lacks significant cellulolytic activity, lignocellulosic material should be submitted to a pretreatment, *i.e.* hydrolyzed, to release free sugars and increase the conversion to lipids.

Table 1.6 presents a brief review of typical oleaginous yeasts used by several researchers for single-cell oil production using agroindustrial wastes as carbon sources.

Table 1.13: Oleaginous yeasts species for single-cell oil production using lignocellulosic residues.

Oleaginous yeast	Substrate
Cryptococcus sp.	Glucose and corncob hydrolysates (Chang et al., 2015)
Lipomyces starkeyi	Olive mill wastewater (Yousuf, A.; Pirozzi, 2009)
Rhodosporidium toruloides	Crude glycerol (Leiva-Candia et al., 2014)
Rhodotorula mucilaginosa	Potato hydrolysate (Marova et al., 2012)
Yarrowia lipolytica	Sugarcane bagasse and rice bran hydrolysates (Tsigie et al., 2011; Xavier and Franco, 2014)

The main component of the storage lipids are triacylglycerols composed of long-chain fatty acids with an average of 16–18 carbon atoms (Rakicka et al., 2015) mainly polyunsaturated such us oleic, linoleic, linolenic and palmitoleic acids as well as stearic and palmitic acids (Sitepu et al., 2013). The dominant fatty acids are thus similar to those found in plant-derived oils, making them an appropriate substitute for vegetable oils for biodiesel and other oleochemicals.

Additionally to lipids accumulation, certain oleaginous yeasts are also able to accumulate carotenoids. There are a great variety of carotenoids with more than 600 different compounds normally synthetized by plants, bacteria, yeast and algae (Stahl and Sies, 2003). Increasing commercial interest on carotenoids is related with their potential benefits for human health (Frengova and Beshkova, 2009). Despite the availability of a variety of natural and synthetic carotenoids, the research interest on carotenoids production from microbial sources has increased due to restricted rules and regulations currently applied to chemically/purified pigments. Among these sources, *Rhodotorula sp.* is a well known carotenoids producer (Frengova and Beshkova, 2009)(Cutzu et al., 2013).

1.9.1. Lipid accumulation in oleaginous yeast

Accumulation of lipids is a biological process that fulfills several roles in microorganisms. Lipids storage contributes to cell growth, cell division, stress response, and as energy storage for survival (Chapman et al., 2011). Most oleaginous yeast species are obligate aerobes and can assimilate a broad variety of carbon sources, an advantageous skill in aqueous, sugar-rich environments. Additionally, they are able to use and store carbon compounds to ensure survival, even in poor-carbon medium. The combination of these characteristics allows yeast to survive for long periods in nutrient-poor niches and feast and store reserves when an unpredictable range of carbon sources, such as lignocellulosic breakdown products, becomes available (I. R. Sitepu et al., 2014). Nitrogen starvation, when carbon sources are still available, is known to trigger lipids accumulation in most oleaginous yeasts (Rattray, 1988).

Lipid profiles as well as the proportions of membrane *vs* storage lipid varies among species and strains, culture conditions such as carbon source (Easterling et al., 2009), nitrogen depletion, age of culture and culture growth phase (I. R. Sitepu et al., 2014).

Generally, lipid accumulation takes place endogenously through one of two pathways: the *"de novo"* and *"ex novo"* processes (Abghari and Chen, 2014). One of the main differences between both pathways relies on substrate used.

De novo lipid accumulation pathway uses hydrophilic substrates such as polysaccharides, organic acid, ethanol, among others, and lipids are synthetized from acetyl-CoA and malonyl-CoA building blocks. In this case, lipid production is a secondary anabolic activity occurring at nutrient-limited (for

instance nitrogen) culture conditions. During *de novo* lipid synthesis, the initiation of lipid accumulation is induced by the exhaustion or a limitation of a primary nutrient from the culture medium.

When nitrogen becomes unavailable, since it is an essential nutrient for protein and nucleic acid synthesis, cell proliferation slows down. However, yeast continues to assimilate the carbon source from the medium, which is now channeled towards lipid synthesis. Growth deceleration will consequently slow down the tricarboxylic acid (TCA) cycle, resulting in an overproduction of citrate, the immediate precursor of the acetyl-CoA in the cytoplasm. Cytosolic fatty acid synthase (FAS) uses acetyl-CoA and malonyl-CoA to synthesize fatty acids, which are then esterified with glycerol generating structural (*e.g*. phospholipids) and reserve lipids (mainly triacylglycerides). Usually, FAS produces the 16-carbon palmitic acid, which can undergo further elongation or desaturation, depending on the enzymatic arsenal of each organism, leading to different TAGs accumulation and LB enlargement. In parallel, a small fraction of the FA is esterified to a sterol to produce the steryl esters. This neutral lipid fraction, stored inside the LBs, can be mobilized depending on the energy requirements of the cell (Beopoulos and Nicaud, 2012). During *de novo* lipid synthesis, the initiation of lipid accumulation is induced by the exhaustion or a limitation of a primary nutrient from the culture medium.

In contrast, the *"ex novo"* lipid production pathway is based on hydrophobic substrates assimilation such as vegetable oils, fatty by-products, among others, and involves the uptake of fatty acids, oils and TAG from the culture medium and their accumulation in an unchanged or modified form within the cell. The latter requires hydrolysis of the hydrophobic substrate and incorporation of the released fatty acids by active transport inside the microbial cell (Papanikolaou, S.; Aggelis, 2011). The synthesized or imported lipid can then be used for growth needs or become a substrate for endocellular biotransformations into other lipid-derived metabolites (Beopoulos and Nicaud, 2012). Therefore, *ex novo* lipid production is a growth-associated process occurring simultaneously with cell growth, being independent from nitrogen exhaustion.

Although the use of alternative and renewable resources as carbon source such as agro-residues for single cell oil production has the potential to be a feasible strategy for cost-effective lipid fermentation with oleaginous yeast but also for other oleaginous microorganisms on a large scale, two main barriers need to be overcome. The first relies on the fact that several oleaginous microorganisms do not easily assimilate some of the main components of the lignocellulosic structure. The microorganisms that are potentially useful for bioconversion must efficiently utilize both hexoses and pentoses, which is not accomplished by most microorganisms. More concretely, the highly efficient utilization of lignocellulosic biomass for the production of valuable chemicals depends on obtaining microorganisms that are capable of using pentoses as a carbon source, which can be promoted by

initially screening or by mutagenesis using traditional or genetic engineering methods (Huang et al., 2013). Therefore, finding a strain able to assimilate or ferment pentoses is critical for any bioconversion that uses lignocellulosic biomass as feedstock.

The second problem is related with the fact that oleaginous microorganisms growth can be hindered by inhibitors that are present in lignocellulosic hydrolysates (Huang et al., 2011)**.** Different methodologies can be applied to overcome inhibitors presence in the medium such as adaptation or genetic modification increasing oleaginous yeasts tolerance to their presence (Parawira, W.; Tekere, 2011). Studies on the effect of inhibitors on the growth and on the accumulation of metabolites could direct the process optimization towards strain improvement (Caspeta et al., 2015)

1.9.2. Carotenoids: synthesis and properties

Carotenoids are the most widely distributed natural class of pigments, displaying yellow, orange and red color, on many fruits, vegetables, some fishes as salmon or trout and crustaceans (Aksu and Tuğba Eren, 2005). They are organized in two main groups: carotenes and xanthophylls according to their chemical structures. Some carotenes such as β -carotene and torulene only have carbon and hydrogen on their chemical structure while xanthophylls such as astaxanthin and canthaxanthin also contain oxygen (McDonald, 2003). These natural pigments have a wide range of market applications such as food coloring agents, e.g. margarine, soft drinks and baked goods; as precursors of vitamin A in food and animal feed; as additives to cosmetics, multivitamin preparations and, more recently, with biological activity as antioxidants with potential benefic effects on degenerative and cardiovascular diseases, on arteriosclerosis, cataracts and multiple sclerosis. (Bauernfeind, 1981; Krinsky, 2003).

Industrially important carotenoid pigments such as β –carotene and astaxanthin, can be produced by yeast (Mata-Gómez et al., 2014) β -carotene has been used as food colorant or as food supplement acting as provitamin A, in concentration ranges from 2 to 50 ppm. It is also added to juices and drinks formulations (hydrophilic matrices) and others (lipophilic matrices) such as butter, margarine and cheese (Miura et al., 1998). Astaxanthin is a red pigment that causes coloration in marine invertebrates, fish and birds. Likewise carotenes are applied as a colorant to confer the typical pink-red color of farmed salmon, trout and shrimp (Johnson and Lewis, 1979). Another important carotene is torulene (ψ-carotene), which has 13 conjugated double bonds, and has a red attractive color. The antioxidant properties of torulene are attributed to its conjugated double bond system. In fact, torulene has higher antioxidant efficiency than β -carotene, which presents less double bonds (Sperstad et al., 2006).

Carotenoids are naturally occurring lipid-soluble pigments, the majority being C_{40} terpenoids, which act as membrane-protective antioxidants scavenging O_2 and peroxyl radicals. Carotenoids market was estimated at nearly $1.2 billion in 2010, but the expectations for 2018 are increasing considerably

supposing to reach $1.4 billion with a compound annual growth rate of 2.3% (Aman et al., 2005). More specifically, β-carotene as a high value compound showed a global market value of $ 233 million dollars in 2010, and is expected to reach $ 309 million by 2018 with an annual growth rate of 3.6% (Mata-Gómez et al., 2014).

There are some species from genus *Rhodotorula*, such as *Rhodotorula glutinis*, *Rhodotorula minuta*, *Rhodotorula mucilaginosa*, *Rhodotorula acheniorum* and *Rhodotorula graminis*, which are recognized as high producers of β –carotene and, therefore, the most studied in that respect. Besides β-carotene these yeasts produce also Torulene, Torularodine, and γ –carotene (Goodwin, 1980; Perrier et al., 1995; Tinoi et al., 2005). The amount of carotenoids produced by *Rhodotorula* species can be classified as low if less than 100 μg g^{-1}, medium if in between 101 to 505 μg g^{-1} and high if higher than 500 μg g^{-1} (Davoli et al., 2004; Mata-Gómez et al., 2014).

Currently, carotenoids are obtained by chemical synthesis or by extraction from plants and/or algae. The chemical synthesis generates hazardous wastes raising environmental issues and the extraction from natural sources have some weaknesses related with the seasonality and geographic variability, which is difficult to control. Biotechnological production of carotenoids represents a feasible alternative if, by using cheap industrial by-products as nutrient sources, the production cost could be minimized. Moreover, carotenoids production by yeast presents several advantages such as safety, reduced production space, independence of environmental conditions and significant carotenoids production yields (Mata-Gómez et al., 2014). Several researchers have been studying carotenoids production from various agro-residues such as sugarcane molasses, whey and grape must, among others, with a significant wide range of yields achieved. Some examples are given in table 1.7.

Table 1.14: Using agro-industrial wastes as substrates to yeast carotenoid production.

Reference	Substrate	Yeast	Yield
(Marova et al., 2012)	Potato	*Rhodotorula mucilaginosa*	56 $mg.L^{-1}$ of β - carotene
(Bhosale and Gadre, 2001)	Sugarcane molasse	*Rhodotorula glutinis*	185 $mg.L^{-1}$ of total carotenoids
(Buzzini, 2000)	Grape must	*Rhodotorula glutinis*	1.1 $mg.L^{-1}$ of β - carotene
(Yimyoo et al., 2011)	Crude glycerol	*Rhodosporidium paludigenum*	3.42 $mg.L^{-1}$ of total carotenoids
(Marova et al., 2012)	Whey	*Rhodotorula glutinis*	46 $mg.L^{-1}$ of β - carotene

Biotechnological synthesis of carotenoids varies between species and is strongly influenced by many factors involved in the processes that can affect the yields and operation costs. Some of those factors are: (1) carbon source, since yeasts metabolism depends on the carbon source (2) light incidence, because microorganisms need to protect themselves from the light which can causes damage and carotenogenesis as a photoprotective mechanism (3) temperature, since it affects cell growth and metabolite production adapting the biosynthetic pathways, including carotenogenesis; (4) aeration, since carotenogenesis is an aerobic process and the airflow rate in the yeast culture represents an essential factor for substrate assimilation and growth rate, cell mass and carotenogenesis (Mata-Gómez et al., 2014). Moreover, carbon and nitrogen sources influence the carotenoid profiles (Marova et al., 2011). The composition of the nutrient resource is very important to define the preparation of culture media to improve the carotenogenesis on microorganisms.

1.10 Biodiesel production from alternative oil sources

Lipids produced by oleaginous yeasts can be used as an alternative lipid source for biodiesel, presently produced from palm oil, rapeseed or soybean derived lipids (Santamauro et al., 2014). However, these crops compete with food industry for agricultural land and consequently have both a negative public image and environmental impact (Fargione et al., 2008).

As part of the Paris Climate Conference outcomes, EU is committed to the reduction of emissions by 40% and boost renewable energy by 27% until 2030. To achieve these goals, the transition from fossil fuels to bio-based fuels using sustainable sources is of the utmost importance (Juncker and President,

2015). Biodiesel is viewed as a greener alternative to fossil fuels, mainly due to the reduction of noxious exhaust emissions (Demirbas, 2009a). Biodiesel can be regarded as part of the solution to meet the EU goals (Abbaszaadeh et al., 2012; Atabani et al., 2012; Demirbas, 2009a).

One of the main issues that hinder the implementation of biodiesel is its high production cost, feedstock being responsible for about 75% of the biodiesel production cost. Moreover, the oil source is also related to the sustainability of biodiesel. The high biodiesel market demand creates a tremendous toll on the use of farmland necessary for the production of oleaginous crops which will increase food prices (Demirbas, 2009a). This controversy of food vs. fuel has also delayed the implementation of biodiesel and its acceptance by the general public. Alternative sources of oil that do not compete with food industry must be explored. Recently, much attention has been paid to the exploitation of microbial oils. Most of them present lipid profiles similar in type and composition to plants and could therefore have many advantages since they do not compete with food industry. Among microorganisms, yeasts seem to be very promising as they can be genetically manipulated and are suitable for large-scale fermentation (Beopoulos and Nicaud, 2012).

Biodiesel is a mixture of alkyl esters of long-chain fatty acids derived from renewable feedstocks, such as vegetable oils or animal fats, after the transesterification of triacylglycerols with a short chain alcohol, methanol or ethanol, to produced fatty acid alkyl esters (biodiesel) and glycerol (Abbaszaadeh et al., 2012).

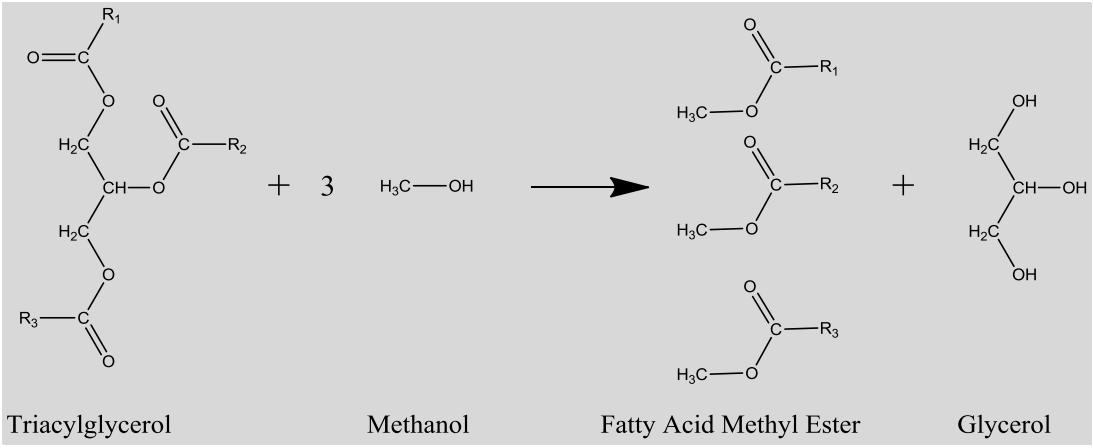


Figure 1.38: Schematic of the transesterification of triacylglycerols with methanol to produce fatty acid methyl esters (FAME - biodiesel).

Homogeneous alkaline catalysis using sodium or potassium hydroxide and acidic catalysis using sulphuric acid as catalysts are still the preferred routes for the industrial production of biodiesel (Abbaszaadeh et al., 2012; Atabani et al., 2012; Talebian-kiakalaieh et al., 2013). Nevertheless this route presents several drawbacks as the formation of soaps when using oils with a high content in

FFA, which consumes the catalyst, decreases yield and makes the purification of biodiesel difficult as well as the high environmental impact from the use of large volumes of water to wash the biodiesel.

Enzymes do not form soap and can convert both FFA and TAG. Moreover enzymes work at mild condition lowering energy requirements. There is also no need for complex downstream processing and cleaning of fuel, therefore a high purity biodiesel and glycerol can be easily obtained. The high purity of glycerol is of the utmost importance for the reduction of the overall biodiesel production cost (Yang et al., 2012). Several lipases and mixture of lipases, e.g., *Pseudomonas fluorescens*, *Candida rugosa*, *Rhizomucor miehei*, *Thermomyces lanuginosus*, *Candida antarctica*, have been used in the conversion of oil from different sources into biodiesel with yields above 90% (Fernandez-Lafuente, 2010; Fjerbaek et al., 2009; Liu et al., 2010; Rodrigues et al., 2011). Recently, it has been claimed that this technology has been applied in a biodiesel plant in China, with a capacity of 20,000 tonnes/year (Robles-Medina et al., 2009). The enzymatic transesterification of triacylglycerols can be carried out in solvent-free systems. Nevertheless and although high yields can be achieved, the extent of diffusion limitations caused by the high viscosity of the reaction medium and the inhibition caused by excess methanol and products may lead to low reaction rates (Azocar et al., 2010; Modi et al., 2007; Ognjanovic et al., 2009; Samukawa et al., 2000; Soumanou and Bornscheuer, 2009; Xu et al., 2003). A molar excess of alcohol over triacylglycerols is required to reach high yields of transesterification, but this excess does not favour enzyme integrity, therefore compromising its operational stability. Due to the high viscosity of vegetable oil, organic solvents are often used as reaction medium to decrease mass transfer limitations. Methanolysis conversions as high as 97 % in *tert*-butanol have been reported using immobilized *Candida Antarctica* lipase B – Novozym 435 (Hernández-Martín and Otero, 2008; Modi et al., 2007; Royon et al., 2007) – in batch or fed-batch mode. The latter approach aims at alleviating the deleterious effect of methanol on the enzyme (Soumanou and Bornscheuer, 2009). Alternatives include using an acyl acceptor with a lower deleterious effect on the enzyme, i.e. ethanol or methyl acetate (Du et al., 2008; Ognjanovic et al., 2009; Rosa et al., 2009). However, methanol is cheaper, more reactive, and the fatty acid methyl esters (FAME) formed in the reaction are more volatile and have a lower viscosity than the alkyl esters that result from using other acyl acceptors. Using other enzymes is another option. Immobilized lipase from *Thermomyces lanuginosus* – Lipozyme TL IM – has been proven to be a viable alternative to Novozym 435, in some cases promoting higher reaction rates in the presence of higher loadings of methanol (Hernández-Martín and Otero, 2008; Wang et al., 2008). Mixtures of the two enzymes have also been used (Li et al., 2006).

To further address environmental concerns, organic solvents can be replaced with supercritical CO_2 ($scCO_2$) (Wen et al., 2009). $ScCO_2$ is able to diffuse through solid matrices, reducing internal diffusional limitation in supported enzymes, and external diffusional limitations by improving

diffusivities of the dissolved components (Matsuda et al., 2005a). Moreover by simple manipulation of temperature and pressure the solvation power of $ScCO_2$ can be easily adjusted (Jackson and King, 1996; Reverchon and Marco, 2006). This allows for a significant simplification in the fractionation of the reaction products thus reducing downstream costs. Also, CO_2 can be completely separated from the reaction products by simple depressurization and recycled to avoid greenhouse gas emissions (Jackson and King, 1996; Rodrigues et al., 2011; Rosa et al., 2009; Varma et al., 2010).

The economic impact of biocatalysis is apparently a disadvantage, when considering the cheaper alternative of alkaline catalysts. But it may not be so if a $ScCO_2$-based integrated reaction/separation process is designed, whereby a stream of oil-rich $ScCO_2$ is fed to a bioreactor for conversion of the triacylglycerols into biodiesel, followed by a facilitated downstream separation process that the use of a $ScCO_2$ ensures. $ScCO_2$ can selectively extract/recover the alkyl esters from the reaction mixture since the solubility of the esters in $ScCO_2$ is several orders of magnitude higher than the solubility of triacylglycerols and glycerol. For instance, at 60 ºC and 14 MPa, the solubilities of methyl palmitate and methyl oleate in $ScCO_2$ are, respectively, 41 and 20 g/kg_{CO2} (Inomata et al., 1989), while the solubilities of triolein and glycerol are, respectively, 1.0 and 0.06 g/kg_{CO2} (Sovová et al., 1997). This shows that $ScCO_2$ can selectively extract the alkyl esters from the non-converted triacylglycerols and by-product glycerol. Moreover, the use of enzymes has the economical advantage of yielding high grade glycerol with market value, unlike the alkaline process. Also the enzymatic process operates at lower temperatures (between 20 and 50 ºC; 60-80 ºC for the alkaline process), which translates into energy savings. In addition, the alkaline process requires an intensive wastewater treatment, with a high environmental impact, a situation that does not occur in the enzymatic process.

In this work, in order to evaluate the viability of this technology in the production of biodiesel from alternative sources of oil, waste cooking oil (WCO) was used. WCO, which is a residue without commercial value, has an annual production of about 29 million tons (Enweremadu and Rutto, 2010). As with yeast oil, WCO triacylglycerols are mainly composed by palmitic, stearic, oleic, linoleic and linolenic acids (Sitepu et al., 2013)(Maddikeri et al., 2012).

1.11. Thesis outline

The main goal of this study was the design of an integrated bioengineering system capable of creating a valorization chain for lignocellulosic residues. The process diagram is represented in figure 1.39.

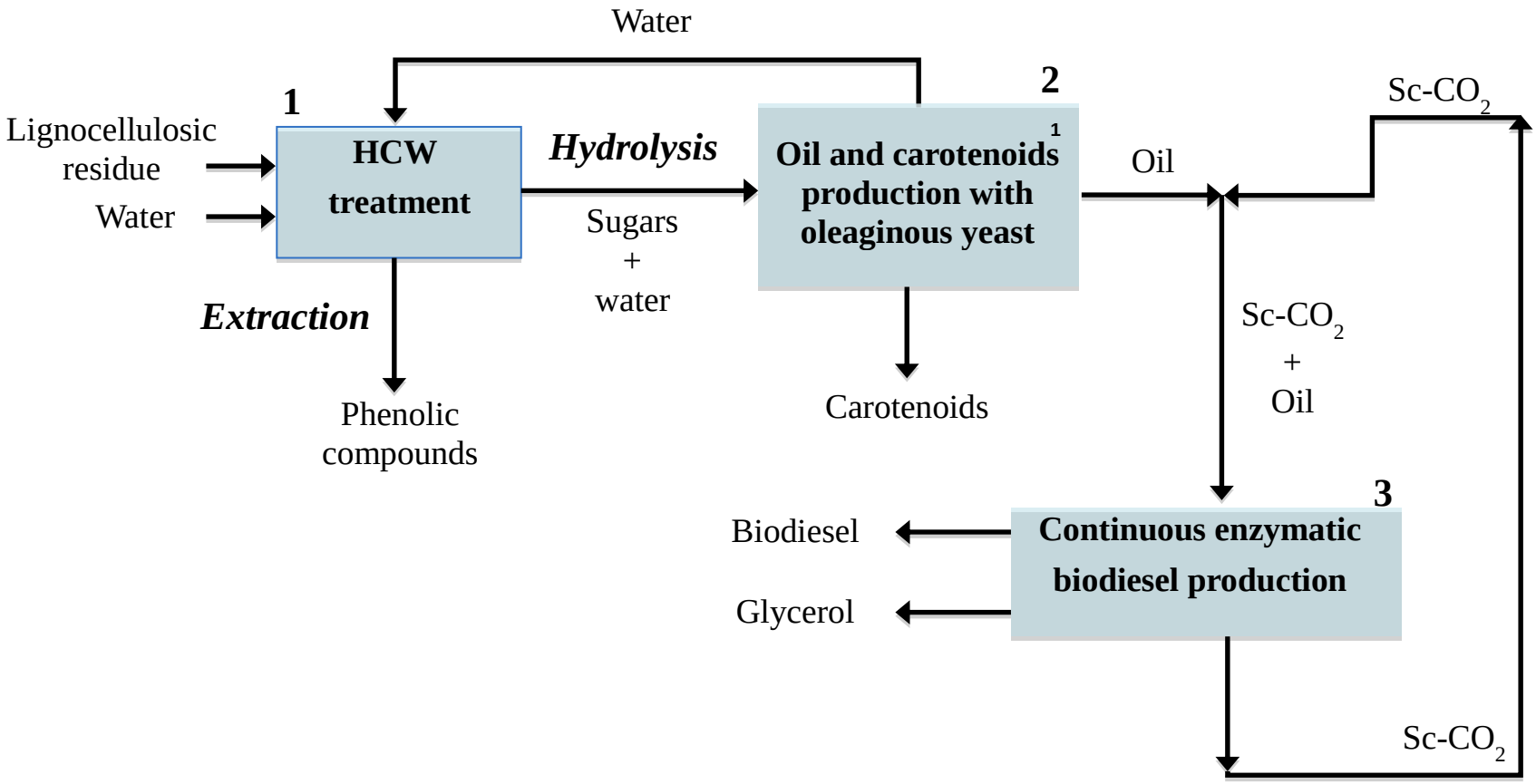


Figure 1.39: Diagram for the proposed integrated bioenginnering system for lignocellulosic residues valorization chain.

In summary, HCW will be evaluated for the simultaneous extraction of added value compounds such as phenolic compounds, as well as for the hydrolysis of the main structural components present in the lignocellulosic residues matrixes studied (1); the sugar-rich aqueous solutions obtained will be used as single carbon source for oleaginous yeast growth accumulating added value products such as lipids and carotenoids (2); the yeast oil can be used directly or as feedstock for the production of biodiesel using an integrated continuous system combining Sc-CO_2 and enzymes.

This thesis is organized according to the following chapters:

- **Chapter 1:** Describes the motivation as well as the background of the main concepts applied in this study;
- **Chapter 2:** Describes all the materials and methods used including the apparatus used for the experiments development.
- **Chapter 3:** Include a detail study about the used of HCW to perform the hydrolysis of a lignocellulosic residue, the recycled paper mill sludge. In this study, two operational modes were studied using a batch and semi-continuous systems. A detailed analysis regarding hydrolysis efficiency as well as mass balances were performed to evaluate the potential of each system in the proposed integrated bioengineering process.
- **Chapter 4:** HCW treatment was evaluated in the hydrolysis of grape pomace, as well as in the simultaneous extraction of phenolic compounds present in this residue. Moreover, an initial approach was performed to evaluate the potential of using the hydrolysates obtained to perform the growth of yeasts.
- **Chapter 5:** Considering the results obtained in Chapter 4, a more detailed study about the use of the hydrolysates as alternative carbon sources was performed. For this, apple pomace was used. The main goal was not only to evaluate yeast growth but also the accumulation of lipids and carotenoids at a higher scale.
- **Chapter 6:** The use of an alternative oil source to produce biodiesel using an integrated constinuous process combining supercritical fluids technology and enzymes was performed. This work originated a publication in an international peer reviewed journal referenced as: Rodrigues, A.R., Paiva, A., Gomes, M., Simões, P., Barreiros, S., 2011. Continuous enzymatic production of biodiesel from virgin and waste sunflower oil in supercritical carbon dioxide. J. Supercrit. Fluids 56, 259–264. doi:10.1016/j.supflu.2010.10.031.
- **Chapter 7:** General conclusions and future work.

2

Materials and Methods

2.1. Materials

Three lignocellulosic residues were used as raw material for the hot compressed water treatment. Recycled paper mill sludge (RPS) was provided by LNEG- Laboratório Nacional de Energia e Geologia institution. Grape pomace (GP) from the fermentation of red wine was kindly provided by a Portuguese wine producer. Apple pomace (AP) was provided by Birmingham University, UK within collaboration with the Supercritical Fluids Group.

RPS was directly used in the system without any pre-treatment and stored at room temperature. Red grape and apple pomaces original raw materials were freeze-dried for 48 h, allowed to warm to room temperature and milled to ca. 2 mm particle sizes. The resulting powders were stored in plastic bags kept at -18 ºC.

Standards used for methods calibration such us 5-hydroxymethylfurfural (5-HMF; ≥ 99%) and furfural (99%), phenol (99%), chlorogenic acid (99%), gallic acid (99%), resveratrol (99%), caffeic acid (99%), p-coumaric acid (99%), ferulic acid (99%), diethyl ether (≥ 99%), D-glucose, D-Fructose, D-galactose, D-xylose, D-mannose, L-Fucose, L-arabinose, L-rhamnose, sucrose, maltose, cellobiose, maltotriose and Viscozyme L (liquid cellulolytic enzyme mixture), were provided from Sigma-Aldrich. Minimal media Yeast Nitrogen Base (YNB) was provided from Difco™.
All other reagents were of high grade and purchased from available suppliers.

2.2. Lignocellulosic residues characterization

Recycled Paper mill Sludge

RPS as-received chemical characterization was performed by the provider which included lignin, polysaccharides, fat and protein contents. In addition, ashes content was determined by igniting the raw material at 550ºC during 5h (NREL 2014).

Red grape pomace

The average water content and the ash content of GP powder were determined by drying ca. 0.5 g samples at 105 ºC for 4 h, and by ashing ca. 0.5 g samples in a furnace at 550 ºC for 5 h, respectively (NREL, 2014). The amount of nitrogen in GP powder was determined by elementary analysis and the factor 6.25 was used to convert to crude protein amount (NREL, 2014).

The amount of lipids was determined by submitting ca. 2 g of GP powder to Soxhlet extraction for 4 h using *n*-hexane.

Defatted GP powder (0.8 g) was extracted three times in succession with 40 mL of a 80:20 (v/v) ethanol:water solution for 15 min at room temperature in an ultrasonic unit (Deng et al., 2011). After each extraction, the mixture was centrifuged at 10,000 RPM for 10 min, and the supernatant was collected. The combined supernatants from the three extractions were evaporated at 50 °C under vacuum on a rotary evaporator to remove ethanol. Water was then added (80 mL). Glucose, fructose and fucose in this solution were quantified by high-performance liquid chromatography (HPLC) with a Dionex ICS-3000 system, with electrochemical detection, using a 4x50 mm Thermo BioLC Dionex AminoTrap pre-column and a 4x250 mm Thermo Dionex CarboPac SA10 column, at a constant temperature of 40 ºC. A 1 mM NaOH solution was used as mobile phase, at a constant flow rate of 1.2 mL/min. Calibration curves were built for the three monosaccharides (25, 50, 75, 100, 150, and 250 ppm).

Defatted, soluble sugar-free GP powder (0.3 g) was submitted to a two-step acid hydrolysis (NREL, 2014). First with 72% (w/w) sulphuric acid (3 mL) for 1 h, at 30 ºC, in a water bath, and after addition of 84 mL of ultrapure water to achieve a 4% (w/w) solution of sulphuric acid, hydrolysis proceeded for 1 h, at 121 ºC, in a silicone bath. After filtration, glucose, galactose, fructose, arabinose, mannose, xylose and fucose were quantified by HPLC, as indicated above. Standard solutions of these monosaccharides were submitted to treatment with sulphuric acid to correct for losses due to degradation of sugars during dilute acid hydrolysis (NREL, 2014) and 4% (w/w) sulphuric acid was used for dilution to build calibration curves.

The remaining residue after acid hydrolysis was rinsed with water, dried at 105 ºC to determine its dry weight, and the amount of nitrogen was determined by elementary analysis. The factor 6.25 was used to convert to acid resistant protein amount (Deng et al., 2011). The amount of acid insoluble lignin was calculated by subtracting the amount of acid resistant protein and acid insoluble ash from the amount of acid insoluble residue.

2.3. Hot compressed water treatment apparatus

2.3.1. Hot compressed water (HCW) treatment apparatus using a semi-continuous flow reactor

Experiments were performed using a semi-continuous-flow apparatus (Figure 2.1 and 2.2). Water is pre-heated before it enters a 3 mL, stainless steel, tubular reactor where the residue powder is placed between two 10-15 µm filters. Water soluble products are recovered by cooling and depressurizing the outlet stream of the reactor. The temperatures of the inlet and

outlet streams of the reactor and the temperature inside the reactor (working temperature) are measured with PT100 sensors. These are connected to units that control the temperature of the heating jackets of the water pre-heating section and the reactor. Water is continuously pumped with a liquid pump (LDC Minipump, model NSI-33R, maximum flow of 7.5 mL/min). The pressure in the reactor is controlled with a back pressure regulator valve (Tescom 27-1700).

To perform an experiment, ca. 2-3 g of each residue powder are loaded into the reactor, which is then filled with water at ambient temperature and pressurized to 100 bar. The two heating jackets are then turned on, at a heating rate of 20 ºC/min, and water starts being pumped, at a flow rate of 2 mL/min. When the working temperature reaches 50 ºC (after about 5 min), is considered time 0. The whole of the outlet stream (called liquor) of the reactor is collected for analysis, in a set of flasks (14 mL samples), through the duration of the experiment. The first samples are thus taken while temperature is increasing to the desired working temperature (e.g. it takes an additional 15 min for temperature to rise from 50 ºC to a working temperature of 180 ºC). When the liquors cool down to ambient temperature, some precipitation occurs. The liquors are filtered and stored at 4 ºC. The amount of solid remaining in the filter did not exceed 2% of the initial amount of the residue placed in the reactor, and was not analyzed.

All experiments were performed at a constant pressure of 100 bar. Higher flow rates and heating rates were also assayed, as it is described in the next chapters.

The residues after hydrolysis with HCW were washed and dried in an oven at 105 ºC for 4 h, and weighed.

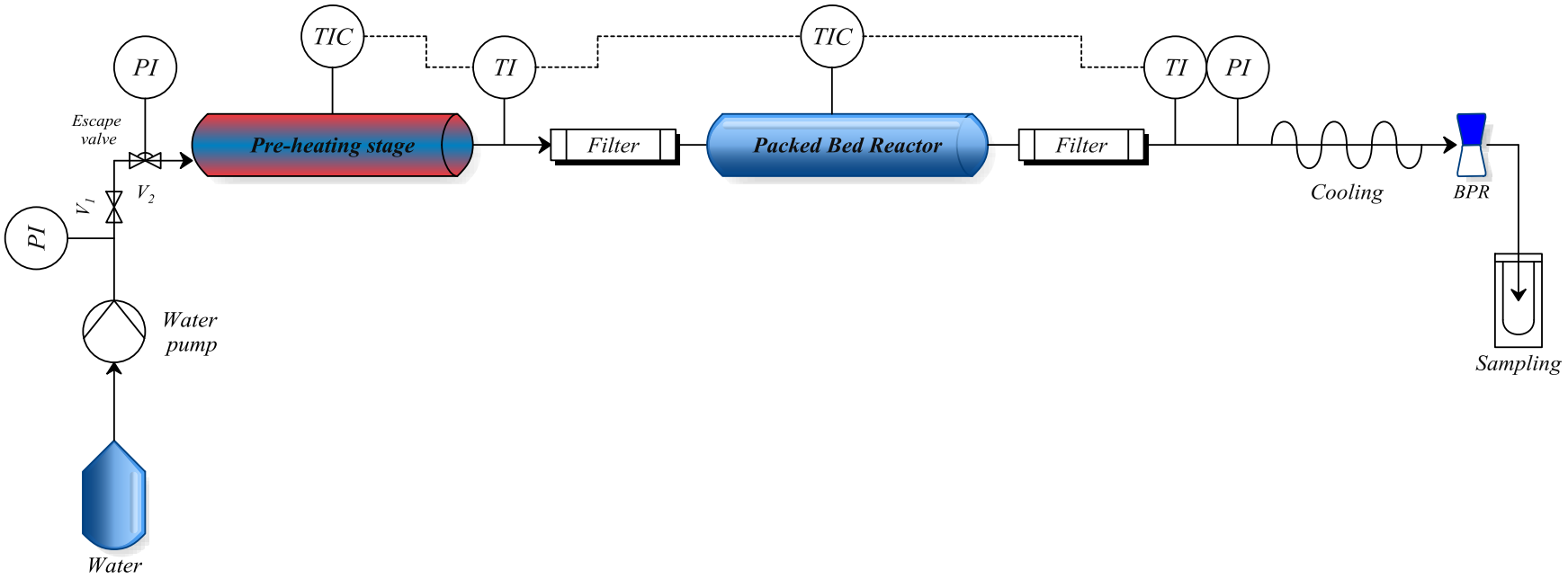


Figure 2.1: Hot compressed water process flow diagram.

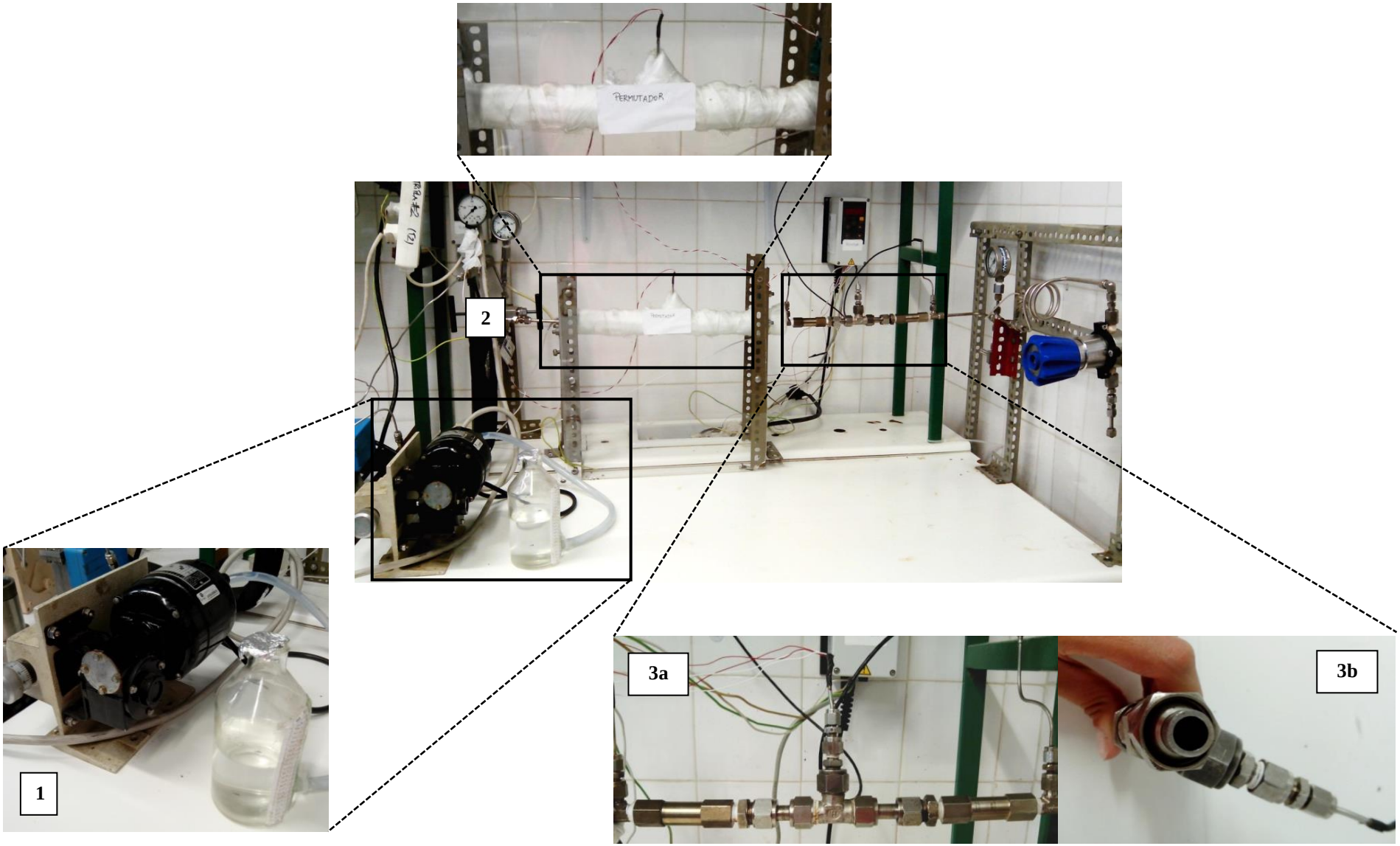


Figure 2.2: Hot compressed water treatment apparatus. 1: Water pump; 2: Pre-heating stage; 3: 3a- Semi-continuous flow reactor, 3b- reactor section.

2.3.2. Hot compressed water hydrolysis of RPS- Batch experimental setup

Experimental results obtained in batch system were performed in collaboration with Birmingham University, Chemical engineering department, Birmingham, UK.

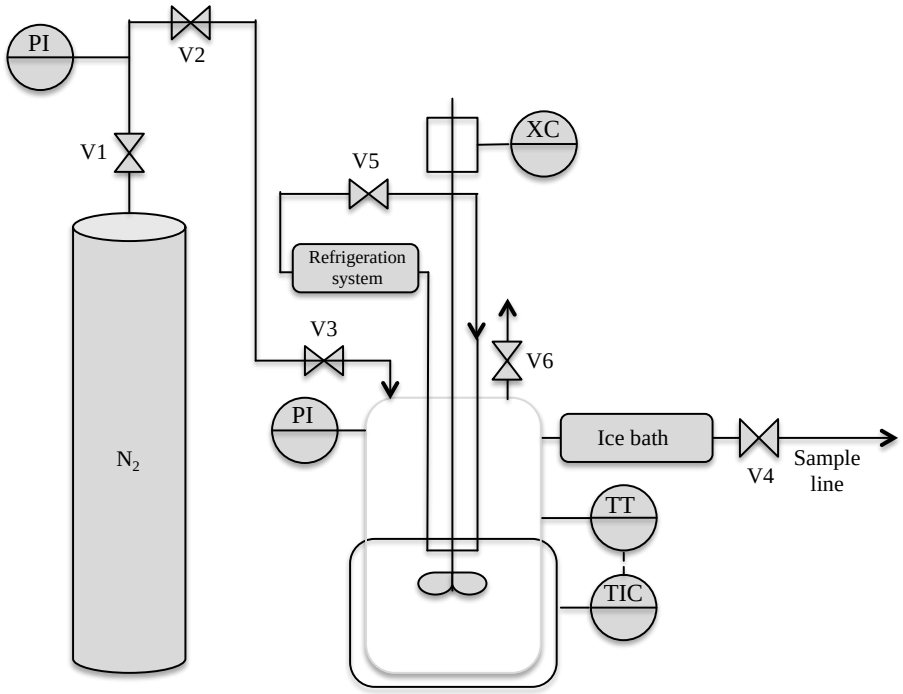


Figure 2.3: Batch experimental set-up diagram (V1-V3: nitrogen supply valves, V4: sample line valve, V5: refrigeration water supply valve, V6: pressure relief valve).

HCW hydrolysis of RPS was performed using a 300 ml stainless steel vessel (Parr Instrument Company, model 5521) and 10g of RPS was initially charged with 200ml of water corresponding to a solid: liquid ratio of 1:5 and two-thirds of the reactor volume. The agitation rotor was set to 1200 rpm. The reactor was pressurized by using a nitrogen gas cylinder and the required pressure of 100 bar was kept constant by controlling the supply of nitrogen throughout valves V3 and V6 operation (Figure 2.3). Reactor temperature was controlled by the heating jacket control system and a cooling loop system using a refrigerator liquid which flow was controlled by V5 manipulation though the experience time.

After RPS charging, the reactor is sealed and placed into the heating jacket. All the valves, excluding the relief valve, are closed. The stirrer rotor is turned on. The vessel is then pressurized to a certain pressure, depending on the desired operational temperature, using the nitrogen line and the relief valve is slowly closed after purging the oxygen trapped inside the vessel. The heater is turned on to the set temperature. The relief valve is adjusted to maintain the pressure inside the vessel at 100 bar. The first sample is collected when the set temperature is

reached (time = 0). Liquors samples of 5 ml were taken at 0, 10, 20, 30 and 60 min of reaction time. After sample collection, pressure was readjusted. Experiments were performed at 150, 180, 200, 220 and 250 °C.

All the samples were centrifuged at 15000 rpm during 10 min at 4 ºC to remove solids suspension before further analysis performance.

2.4. Liquors characterization after HCW treatment

The following parameters were analysed directly from the liquors after HCW hydrolysis: the amount of water-soluble compounds (WSC's), total amount of carbohydrates, total phenolic compounds and degradation compounds quantification.

WSC's- Water soluble compounds quantification

The amount of water-soluble compounds in the liquors was determined by freeze-drying 2 mL of the liquors samples collected throughout the HCW experiments and weighing the resulting powder, henceforth designated as "hydrolysate".

Total carbohydrates colorimetric analysis

The total amount of carbohydrates in the liquors was determined using the phenol-sulphuric acid colorimetric method (Dubois et al., 1956). To 500 µl of liquors samples it was added 1.5mL of H_2SO_4 acid (Panreac 96%) and 300 µl of 5% of phenol (SIGMA Aldrich 99-100%). After incubating for 5 min at 90°C into AccuBlockTM Digital Dry Bath, samples were cooled to room temperature using an ice bath and the absorbance was measured at 490 nm using DU®800 Spectrophotometer from Beckman Coulter, Brea, USA.

Calibration was performed for glucose in ultrapure water considering the following concentrations: 0.005; 0.025; 0.05; 0.1; 0.15; 0.2; 0.25 and 0.3 g/L. The amount of oligosaccharides in the extracts was estimated by subtracting the amount of monosaccharides in the hydrolysates (described in the next section) from the total amount of carbohydrates. An additional correction was made based on the amounts of degradation compounds, determined as indicated in this section, since they interfere directly with the final measurements obtained by this colorimetric method.

Total phenolic compounds quantification

Total Phenolic Content (TPC) was determined using the Folin-Ciocalteu method (Folin and Ciocalteau, 1927). Calibration standard curve was performed with five different concentrations of Gallic Acid monohydrate (Sigma 98%) (50, 100, 150, 250 and 500 mg/L) and for blank solution was used Mili-Q water.

Protein precipitation was performed using 800 µl of each liquor sample and adding 120 µl of Trichloroacetic Acid (Scharlau 99,5%). Samples were stored at -20 ºC for 5 min, then at 4 ºC for 15 min and centrifuge (Heraeus sepatech, Biofuge 13 Centrifuge) for 15 min at 12 000 rpm. To 20 µl of resulted supernatant, it was added 1,58 ml of Mili-Q water, 100 µl of Folin-Ciocalteu reagent (MERK) and the resulted solutions were stored at room temperature for 8 min. Then, it was added 300 µl Sodium Carbonate (Sigma) solution (200 g/L) and incubated in water bath at 40 ºC for 30 min. The absorbance was measured at 750 nm using a spectrophotometer (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA).

Degradation compounds quantification by HPLC analysis

The quantification of degradation compounds – furfural and 5-HMF – directly in the liquors was carried out by HPLC with UV detection at 280 nm (Schieber et al., 2001) using a 250x4 mm, C18 BDS HYPERSIL column, at a constant temperature of 40 ºC. The mobile phase was a mixture of two eluents. Eluent A was a 2% (v/v) solution of glacial acetic acid in ultrapure water, and eluent B was a 0.5% (v/v) solution of glacial acetic acid in a 1:1 (v/v) mixture of ultrapure water and acetonitrile. The gradient program was: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min). Calibration curves were built with ten solutions of either furfural or 5-HMF, with concentrations ranging from 25 to 250 ppm.

Acetic acid quantification by HPLC analysis

The quantification of acetic acid was also carried out directly in the liquors, using a Dionex ICS-3000 system with a PDA ICS Series - 210 nm detector, using a 7.8 x 300 mm BIORAD Aminex HPX-87H column with pre-column, at a constant temperature of 30 ºC. A 10 mN H_2SO_4 solution was used as mobile phase, at a constant flow rate of 0.6 mL/min.

2.5. Monosaccharide analysis by HPLC

Monosaccharides amounts recovered were analyzed trough a previous aqueous solution preparation with the hydrolysates at a concentration of 5000 ppm.

The monosaccharide analysis was performed by HPLC using a Thermo Scientific Surveyor Plus HPLC System with refractive index detector and HyperRez XP Carbohydraten Ca2+ (300x7.7 mm) column at constant temperature of 85 ºC. Ultrapure water was used as the mobile phase at a constant flow rate of 0,6 ml/min.

For a more accurate analysis, the method of standard addition calibration was used where 100 ppm of internal standard is added directly to the aliquots of analyzed sample. Therefore, the total concentration of the analyte is the combination of the unknown amount and the100 ppm of the internal standard .The standard addition method is an effective method to determine the concentration of an analyte in the presence of other chemicals that interfere with its analytical signal (Miller and Miller, 1988). Standard curves were performed for glucose monohydrate, fructose, arabinose, xylose and galactose (25; 50; 75; 100; 150; 250 and 500 ppm) with 100 ppm as internal standard concentration. All the samples were filtered using syringe NY filters (0,20 µm GVS SpA).

2.6. Residues treatment after HCW treatment analysis

The remaining residues after RPS and red grape pomace hydrolysis with HCW were submitted to a two-step acid hydrolysis with sulphuric acid, same procedure as it was described above in Red Grape Pomace chemical characterization section. The remaining residue after acid hydrolysis was rinsed with water, dried at 105 ºC to determine its dry weight. The amount of acid insoluble lignin was calculated by subtracting the amount of acid resistant protein and acid insoluble ash from the amount of acid insoluble residue.

2.7. Phenolics compounds extraction

2.7.1. Phenolics compounds extraction from GP using organic solvents

Three conventional solvents were used to evaluate the initial phenolic compounds content of GP.

An hydro-alcoholic extraction was performed using 1g of GP residue and adding 20 ml of a mixture of water: ethanol (75:25 v/v) during 18h at 50 ºC with a constant magnetic agitation of 150 rpm.

Two consecutive extractions using 10 ml of a mixture containing water: acetone (20:80 v/v) were performed to 1g of GP for 15 min in an ultrasonic bath at room temperature and then stirred for 30 min on a magnetic agitation. After centrifugation at 3000 rpm for 10 min, both collected supernatants were combined and filtered.

An extraction using 1 g of GP and aqueous solution of citric acid (3g/L) was performed by adding 10 ml of citric acid solution for 30 min at 40 ºC with a constant magnetic agitation (150 rpm). The extract was then filtered and further analyzed.

Samples preparation for HPLC analysis:

To all samples containing the organic extracts obtained from the methods described above it was added 5 ml of diethyl ether and stored at room temperature under constant magnetic agitation of 150 rpm for 10 min. This procedure was repeated 3 times. The obtained organic phase was firstly dried using a Na_2SO_4 and the solvent was evaporated under nitrogen flow. The dried extract obtained was dissolved in a solution of methanol: water (1:1 v/v) and filtered with NY Filters (0,20 μm GVS SpA). Further HPLC analysis was performed as described below.

2.7.2. Phenolic compounds quantification by HPLC analysis

The identification and quantification of individual phenolic compounds present GP extracts was performed using a Thermo Scientific Surveyor Plus HPLC System with a UV detector and BDS HYPERSIL C18 column (length 250mm; I.D. 4mm) at a constant temperature of 40 ºC.

The mobile phase is a mixture of two eluents, A and B. Eluent A is ultrapure water with 2% (v/v) of acetic acid glacial (Scharlau 99,8%) and eluent B is a mixture of water and acetonitrile (CALO ERBA 99,9%) (50:50) with 0,5% (v/v) of acetic acid. The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min).

Standard curves were performed to 5-HMF (25; 50; 100; 150; 200; 300; 500 and 1000 ppm), Furfural and Chlorogenic acid, (25; 50; 75; 100; 150; 200; 250; 300; 500 and 1000 ppm) Gallic acid (50; 100; 200; 300; 400; 500 and 1000 ppm), Resveratrol (5; 10; 15; 20; 25 and 30 ppm), Caffeic acid and p-Coumaric acid (25; 50; 75; 100; 125; 150; 200 and 250 ppm), Ferulic acid (50; 100; 150; 200; 250 and 300 ppm) in ultrapure water.

The analysis of the amount of phenolic compounds extracted using HCW from each experiment was performed by using a water solution with the amount of hydrolysates obtained after liquors freeze-drying at 5000 ppm which would allow an accurate HPLC analysis. All the samples were filtered using syringe NY filters (0,20 µm GVS SpA).

2.8. Yeasts growth

All the yeasts strains were from the Portuguese Yeast Culture Collection (PYCC-UCIBIO) and are described in table 2.1.

Table 2.1: Yeast strains

Species	Substrate origin	PYCC number
Rhodotorula Yarrowii	Strawberry tree fruit	5629
Rhodotorula babjevae	Leaves	4781
Saccharomyces cerevisiae	Wine lees from a distillery	4889
Rhodotorula toruloides	Wood	4416
Rhodotorula glutinis	Sea water	4107
Rhodotorula mucilaginosa	Water	4760
Yarrowia lipolytica	Olives	4936

Yeast strains were routinely maintained at 4°C in Yeast Mold agar containing, 0.3% malt extract, 0.3% yeast extract, 0.5% peptone and 1% glucose, 2% (p/v) agar.

2.8.1. Pre-inoculum

Yeast strains were grown under constant agitation at 120 rpm at 25°C for 24h on a medium containing (per liter) 6.7 g of Yeast Nitrogen Base (YNB, DifcoTM) minimal medium and 5 g of glucose. The culture medium components were sterilized by filtration.

2.8.2. Yeast growth using microplates and red grape pomace hydrolysates as carbon source

Three yeast, *R.yarrowii, R. babjevae* and *S.accharomyces cerevisiae,* were assessed for its ability to assimilate a mixture of pure hexoses (glucose, fructose, galactose) and pure pentoses (arabinose, xylose) identified in the red grape pomace extracts, combined in different proportions. The screening was performed in 96-well microplates. The YNB medium contained 5 g of the carbohydrates under study. The culture medium components were sterilized by filtration.

All the growth experiments were performed at 25 ºC for 2 days, with shaking at 120 rpm. Yeast growth was monitored by optical density (OD) measured at 640 nm over time. The data reported are the result of replicate experiments.
These studies were followed by others in which the standard carbohydrate mixture was replaced with GP extract, in such an amount as to yield a concentration of 5 g/L in sugar monomers.

2.8.3. Yeasts growth using apple pomace hydrolysates as carbon source

Five different oleaginous yeasts strains were used in this study: *Rhodotorula babjevae, Rhodotorula toruloides, Rhodotorula glutinis, Rhodotorula mucilaginosa* and *Yarrowia lipolytica***.** Growth experiments were performed in 10 mL test tubes with 1 mL of culture medium (see below) unless otherwise stated, with shaking at 200 rpm and at 25°C. Yeast was inoculated at OD_{640}=0.2 and growth was monitored by OD measured at 640 nm over time. All culture media were sterilized by filtration.

Four different media were tested with the AP hydrolysates as follows:

1. A blend made of all hydrolysates obtained through the HCW hydrolysis of AP performed at temperatures from 50ºC to 250ºC was used as the carbon source in YNB medium, at 12g/L of total monosaccharides, in order to study the potential of AP hydrolysates to sustain yeast growth. Total monosaccharides concentration was calculated based on the HPLC data. A parallel growth control experiment was done with 12g/L glucose.

2. The same blend described in 1. was used in a specific medium, adapted from a medium described by *Zhao et al.* (Zhao et al., 2008) for optimum lipid production in order to identify the best fitted yeast for lipid accumulation. The medium contained 70 g/L carbon source (AP

hydrolysates blend) sterilized by filtration, 0.5 g/L yeast extract, 2 g/L $(NH_4)_2SO_4$, 7 g/L KH_2PO_4, 2 g/L Na_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$.

3. AP hydrolysates obtained at 130 °C, 150 °C, 170 °C and 190 °C were used as the sole carbon source to perform *Rhodotorula mucilaginosa* growth in the specific medium for lipids accumulation described in 2. but now testing each AP hydrolysate in a separate experiment of growth.

4. AP hydrolysate obtained at 130 ºC was used as described in 2. but the growth of *Rhodotorula mucilaginosa* was performed in 10 ml into 50 mL erlenmeyer flasks.

2.8.4. Lipid analysis

For the visualization of lipid droplets, Nile Red (1mg/mL solution in acetone) was added to the cell suspension (1/10, v/v) and incubated for 1 h at room temperature. Cells were then harvested, washed twice with distilled water, resuspended in 50 mM sodium phosphate buffer (pH=6.8) to OD_{640} of 5 and imaged under a fluorescence microscope (Olympus BX51) with a magnification of 100x (Beopoulos et al., 2008).

Lipid droplets semi-qualitative analysis was performed by microscopic observation of cell culture as described above. Ten representative cells were selected from fluorescence microscopy images to determine lipid droplets number for each assay.

2.8.5. Carotenoids extraction and HPLC analysis

Yeast cells at stationary phase (OD…, t= 50h) were harvested by centrifugation the whole culture at 10,000 rpm for 5 min at 25 °C, frozen with liquid nitrogen and freeze-dried. Cells were mechanical disrupted with glass beads in acetone (5 ml) vortexing for 15 min. Carotenoids extraction was performed under agitation during 16h with acetone. Organic solvent was evaporated using nitrogen gas.
Total carotenoids were quantified by spectrometry (DU®800 Spectrophotometer, Beckman Coulter) at λ=452 nm, according to β-carotene extinction coefficient of 485.

A qualitative and quantitative analysis of carotenoids was performed by HPLC (Thermo Scientific, Finning Surveyor AutoSamples Plus) equipped with a reverse-phase analytical 5-µ particle diameter, polymeric C18 column and a UV diode array detector (Accela Uv/Vis Detector). The mobile phase consisted of methanol and 0.2% H_2O/ acetonitrile (75:25 v/v). Total run time was 30 min, with an injection volume of 10 µl. Quantification was performed

with HPLC-DAD (λ = 450 nm) at a flow rate of 1mL/min, using a calibration curve of trans-β-carotene-95% type I, as external standard in the following concentrations 2.5,5,10, 25, 50 ppm.

3

Paper mill sludge HCW hydrolysis

3.1 Introduction

Paper industry is a strategic industry in many countries. However, the production of paper consumes high quantities of energy, chemicals and wood pulp, while generating large quantities of wastewater that has to be purified to avoid environment impact. Pulp and paper mill wastewater treatments typically include a primary treatment consisting of neutralization, screening, and sedimentation to remove suspended solids, which are then dewatered into a sludge that requires disposal. This solid residue is called paper mill sludge and is the major waste generated from paper production and recycling.
Recycled paper sludge (RPS) is generated in large amounts by wastewater treatment facilities of recycled paper plants, representing a serious disposal problem (Koo and Lin, 2004). Since RPS has a significant carbon content, between 28-52%, depending on the waste stream, it could be a possible carbohydrate source for fermentation processes and be further processed to obtain high added value compounds.

The scale up viability of a fermentation process is strongly dependent on the cost of the fermentation medium which can account for almost 30% of the total production cost (Rodrigues et al., 2006). In order to fermentation technologies to be competitive with chemical synthesis it is crucial to find low-cost and highly available raw materials. Therefore, the attention to use lignocellulosic biomass is increasing. The key challenge is to develop hydrolysis technologies that are capable of efficiently recover sugars. Hot compressed water is a non-toxic, non-flammable and environmentally benign solvent to be used for biomass hydrolysis (Möller et al., 2011)(Tahir, 2015).

In this chapter is evaluated the potential of RPS as an alternative carbon source for microorganism growth. Hot compressed water hydrolysis will be used to convert the polysaccharides structure, cellulose and xylan, into their constitutive monosaccharides. Two operations modes for RPS hydrolysis using HCW were studied; a semi-continuous flow reactor and a batch reactor. The influence of operational conditions in the conversion and yield for both processes were assessed.

Severity factor is defined as an empirical measurement of the severeness or intensity of a chemical reaction considering reaction conditions such as temperature, pressure, residence time (Wyman, 2013). The severity factor was firstly derived by Overend and Chornet in 1987 and is based on the assumptions of a first-order kinetics and Arrhenius temperature behaviour of the aqueous pre- hydrolysis of Kraft pulping. The severity factor combines treatment temperature (T) and residence time (t) into a single variable providing an approximate indication of the treatment conditions and is given by (Zhou et al., 2010)

$$logR_0 = log\left[\sum_{i=1}^{n} t_i \times exp\left(\frac{T_i - T_b}{\omega}\right)\right] \quad \text{(Equation 3.1)}$$

where ω is the fitted parameter which is generally assigned to the value of 14.75; t is the reaction time (min); T_i is the operational temperature (ºC); T_b is the base temperature (100ºC); n is the number of pretreatment stages.. In this case, n=1 and the severity factor is given by

$$\log R_0 = log\left[t \times \exp\left(\frac{T_i - T_b}{\omega}\right)\right] \quad \text{(Equation 3.2)}$$

The severity factor has been used to compare and evaluate the severity of different individual steam treatments in the pulping process. Later, it was reintroduced in lignocellulosic materials hydrothermal pretreatments studies (with or without acid) with the same final goal (Pedersen, M., 2010).

A combined severity factor can be used when a dilute acid catalyst is added to the system. In HCW hydrolysis treatments, water behaved as an acid catalyst and for this reason, it can be applied in this study. This new approach has the purpose of subtracting the effect of the pH on the pretreatment severity and is given by (Pedersen, M., 2010)

$$CSF = \log R'_0 = log\left[t \times \exp\left(\frac{T_i - T_b}{\omega}\right)\right] - |pH - 7| \quad \text{(Equation 3.3)}$$

The severity factor or combined severity factor can be used to select operational conditions considering the target system performance.

3.2. Materials and Methods

RPS was provided by LNEG- Laboratório Nacional de Energia e Geologia institution, which also performed the chemical characterization of the raw material. Ash content was determined by igniting the raw material at 550ºC during 5h. Protein content was determined by Kjeldahl method (Hames et al., 2008) using a nitrogen-to-protein conversion factor of 6.25. Fat content by performing a Soxhlet extraction using petroleum ether and cellulose. Xylan and lignin contents were obtained after performing an acid hydrolysis using sulphuric acid.

Experiments were performed using a semi-continuous flow reactor and batch reactor as described in the Chapter 2:Materials and methods.

The solubilized material, Water Soluble Compounds (WSC), oligosaccharides, soluble lignin, acid hydrolysis of the remain RPS residues after HCW hydrolysis, monomers and degradation

compounds quantification were performed as described in the Chapter 2: Materials and Methods.

3.3. Experimental results and discussion

RPS hot compressed water treatment was studied comparing two different operational systems: batch and semi-continuous. The comparison considered several process parameters such as conversion and yield of oligosaccharides, monomers and degradation compounds, among others. Several experiments were performed applying the same range of temperatures and reaction times to both systems in order to evaluate which could be more profitable. All the experiments were performed at 100 bar. Reaction time was considered after operational temperature is reached.

RPS chemical composition, previously characterized, is described in Table 3.1.

Table 3.1: RPS chemical characterization.

Component	*Content (g/100g dried RPS)*
Ash	57.5
Lignin	11.6
Klasson lignin	11.1
Soluble lignin	0.5
Polysaccharides	23.3
Cellulose	18.7
Xylan	4.6
Fat	2.9
Protein	4.7

3.3.1. Semi-continuous water flow rate optimization

Water flow rate is a crucial parameter in the semi-continuous system and it was optimized.

In the semi-continuous process, fresh water is continuously pumped inside the reactor but its contact with the RPS is limited by the residence time resulted from the water flow rate applied. Experiments performed at 250 ºC, 100 bar and 2, 5 and 10 ml/min were performed for water flow rate optimization. The best results were achieved for a flow rate of 5 ml/min, where the highest WSC's content was observed (figure 3.1). At the highest flow rate, 10 ml/min, the lowest amount of WSC was obtained. This was due to the low water residence time. At this flow rate there was not enough time for the complete hydrolysis of high molecular weight structures into smaller and water soluble ones. By increasing the residence time the amount of WSC increases until a water flow rate of 5 ml/min. By further increasing water residence time, degradation reactions start to occur, converting the water soluble compounds into volatile acids and gases, thus decreasing the WSC.

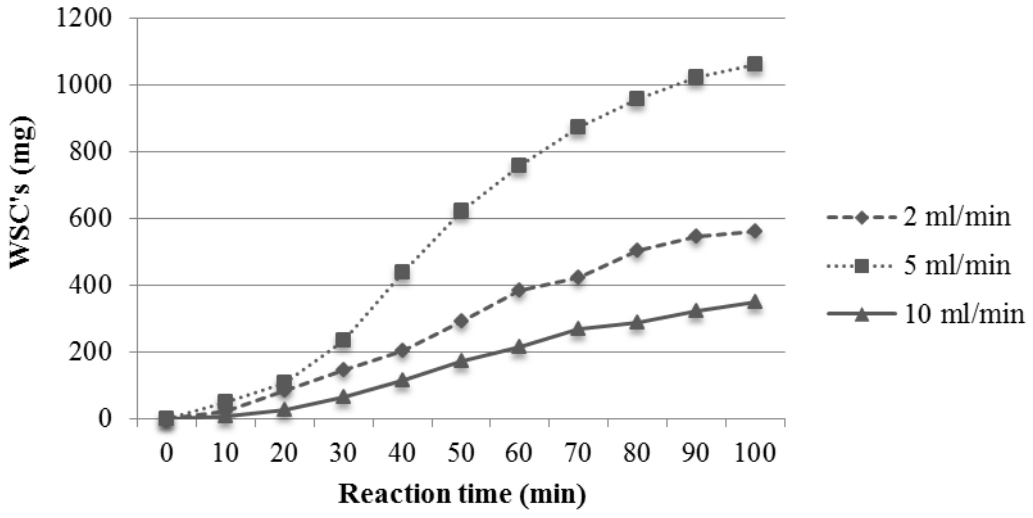


Figure 3.1: Water flow rate optimization. Experiments performed at 250 ºC and 100 bar.

All the experiments of RPS hydrolysis using HCW and a semi-continuous flow reactor were performed at a water flow rate of 5 ml/min henceforth.

3.3.2. Samples pH variation during semi-continuous hydrolysis of RPS

The formation of hydrogen ions represents an important factor to take into account in pressurized hydrothermal treatments. Those ions result from water dissociation and also from organic acids formation due to monosaccharide dehydration. Some authors believe that the formation of organic acids during HCW hydrolysis can facilitate acid-catalyzed hydrolysis of the polysaccharide structure into soluble oligo and monosaccharides due to hydrogen ion liberation (Weil et al., 1998). However, organic acids accumulation at severe conditions, can also promote monosaccharide degradation into byproducts such as HMF, furfural, formic acid and levulinic acid. (Zhou et al., 2010)

The pH during HCW hydrolysis generally varies between 4 and 5 without the addition of a base (Kim et al., 2009). A pH controlled treatment using a base such as potassium hydroxide, could keep the pH within a range of 5 to 7 avoiding acid catalyzed dehydration reactions of monosaccharides (Weil et al., 1998), although this was not applied in the experiments performed during this study.

Analyzing figure 3.2, it can be observed pH variation in all the samples collected during semi-continuous HCW hydrolysis performed at 250 ºC together with WSC's amount to each sample. After 30 min of reaction, pH starts to decrease achieving pH values lower than 5 at the same time that WSC's amount decreases until the end of the experiment. pH values are maintained below 5 for 50 min more, increasing again at the last 20 min of reaction but with values below 6. The pH decreases during all the experiments suggest acid formation resulting from monosaccharides dehydrations reactions. Further details are described in figure 3.3. This theory is supported by the fact that WSC's amount decrease when the pH values decrease to value lower than 5. Since volatile acids are solubilized in the collected samples, pH variation represents a good indicator of degradation reactions extension.

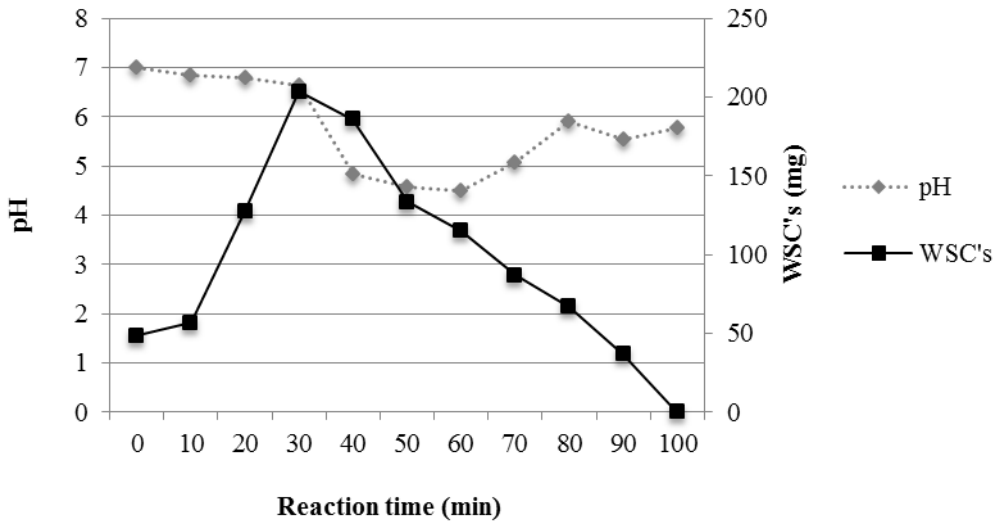


Figure 3.2: Samples pH variation during HCW hydrolysis of RPS performed at 250ºC, 100 bar and 5 ml/min.

In figure 3.3A, it can be observed pH variation for the samples collected during the semi-continuous HCW hydrolysis performed at 150 ºC, 200 ºC and 250ºC.

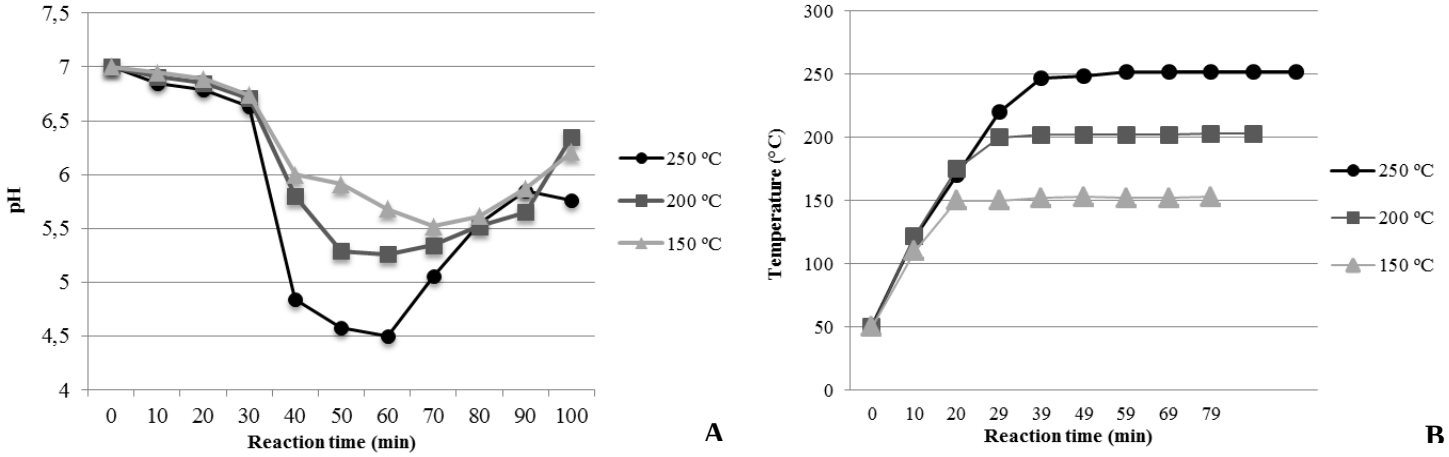


Figure 3.3: pH variation during HCW hydrolysis experiments of RPS performed at 250 ºC, 200 ºC and 150 ºC (A). Experiment temperature profiles (B). All the experiments were performed at 100 bar and 5 ml/min.

For all the experiments, after 30 min of reaction, the pH decreases from 7 to values between 4.5 and 6, depending on the operational temperature. Analysing figure 3.3, the higher the operational temperature (figure 3.3B) the sharper is the pH decrease during the first 30 minutes of reaction (figure 3.3A). After 100 minutes of reaction, water pH remains at values lower than the initial pH. At 250 ºC, pH decreases for the lowest value of 4.5 increasing after 60 min to 5.7.

For the experiments performed at 150 ºC, the lowest value observed was 5.5 and the final sample collect had a pH value of 6.2. Therefore, the expected behaviour was observed: at higher temperature the formation of volatile acids from the consecutive degradation reactions of the monomers (glucose and xylose) is favoured, lowering the pH.

This study was developed in order to evaluate pH variation during the semi-continuous HCW hydrolysis. However, no base was added in order to compare both systems, batch and semi-continuous at the same operational conditions. Further experiments using a base catalyst could be performed to evaluate its influence in monosaccharides recovery.

3.3.3. Process conversion in batch and semi-continuous systems

Process conversion was calculated considering all the organic carbon matter that could be hydrolysed using hot compressed water. Analysing table 3.1, it is considered as hydrolysable material all the components except ashes. Therefore, the carbon matter, representing 42.5% of all matter present on the raw material, includes polysaccharides, lignin, fat and protein. Thus, the maximum conversion that is possible to achieve in each experiment corresponds to this value. The process conversion was determined considering the equation (3.4)

$$Process\ conversion = \frac{g\ Hydrolized\ material}{100g\ RPS} \qquad \text{Equation (3.4)}$$

Considering the hydrolysable material as the amount of mass that is hydrolysed during the process and is calculated by

$$Hydrolized\ material\ (g) = Initial\ mass\ (g) - Mass\ of\ the\ residue\ (g) \qquad \text{Equation (3.5)}$$

- Mass of the residue: amount of mass the remains in the reactor after the experiment
- Initial mass: initial raw material load

From figure 3.4, it can be observed that process conversion increases with temperature in both systems. All other process parameters were maintained constant. Near the critical point, temperature has a more pronounced influence in chemical and physical properties of water. Water dissociation occurs by energy absorption (endothermic process). Considering that endothermic processes are favoured by an increase in temperature, when temperature increases,

the equilibrium moves further to the dissociation of water, which will promote acid hydrolysis. Considering this, hydrolysis reaction rate increases with temperature.

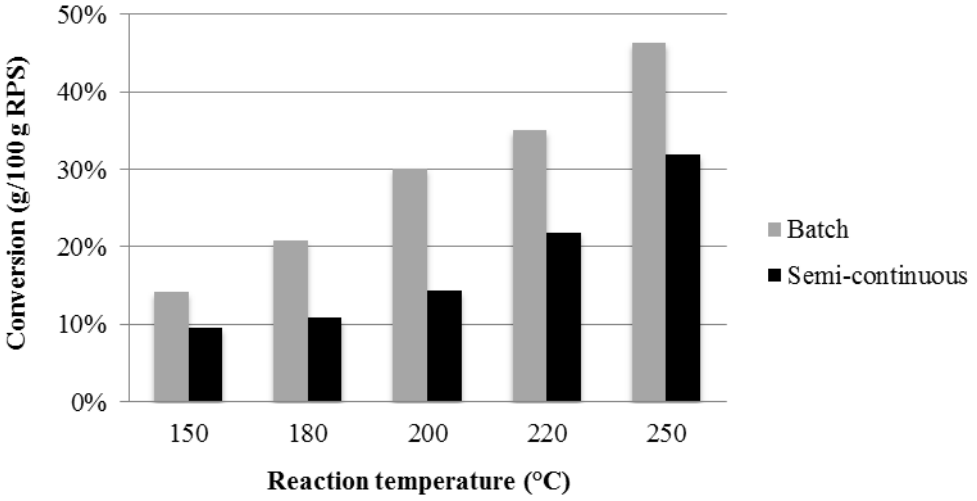


Figure 3.4: Batch and semi-continuous process conversions expressed as g of hydrolysed material per 100 g RPS at 100 bar, 5 ml/min and 1h of reaction time.

It is also very important to notice that several factors influence the polysaccharide complex structure cleavage such us the type and the location of the glycosidic bond within the carbohydrate chain (Sinnot, 2007). For instance, the rate of hydrolysis at the end of the polysaccharide chain is higher since, at this location, the carbon chain is more flexible and more suitable for proton attack (Sinnot, 2007). At higher temperatures, the polysaccharide structure becomes "less longer" due to glycosidic bonds cleavage around the structure turning it more fragile and, subsequently, enhancing its hydrolysis.

Comparing both processes, the batch process achieved higher conversions than the semi-continuous. A conversion of 50% of the maximum possible value was achieved at 180 ºC in batch while it was necessary a much higher temperature (220 ºC) to achieve the same conversion when operating in semi-continuous mode. At 250 ºC, the semi-continuous system achieved 75% of the maximum conversion possible, while in batch this value was 103.5%.

Batch has some characteristics, which can favour hot compressed water hydrolysis. Batch system solid: liquid ratio was 1:5. In a given instant, considering a section of the semi-continuous reactor, the amount of water available per unit of RPS in batch system is higher than in semi-continuous mode, which increases the amount of protons available to perform the glycosidic bonds breakage promoting RPS hydrolysis.

Moreover, stirring of the reaction media plays an important role in reducing diffusional limitations. In batch mode the reaction media was continuously stirred promoting particles dispersion within the water media increasing particle superficial area. In semi-continuous mode, the RPS was packed in the reactor. Therefore, these two operational systems are completely different in terms of solid packing which can easily contribute to different process performances, mainly in terms of conversions and yields. In order to compare both systems regarding particles dispersion inside the reactor, it would be more suitable to perform the comparison with the batch reactor by using a fluidized bed reactor instead of a packed-bed reactor in the semi-continuous system, where more similar reaction conditions would be possibly achieved between both systems. These technical aspects present in batch reactor can justify the reason why RPS conversion was higher in batch system.

Some evidences had already been reported for the solubility of salts such as NaCl or LiCl during near-critical and supercritical hydrothermal treatments. Salts solubility behaviour changes tremendously near the critical point (Tercero, 2014). At relatively low pressures, with increasing temperature, the solubility of salts drops to practically zero. This effect is one of the major drawbacks of processing waste, since salts dissolved in the feed stream could precipitate and eventually block the reactor. However, at more severe conditions of pressure and temperature, salts solubility remains constant or is even increasing (Brunner, 2009). This possibility could explain why the conversion in batch exceeds 100%.

Analysing figure 3.4, it can be observed that conversion increases more linearly in the batch process than for the semi-continuous system. For the semi-continuous system, an eminent increase in conversion with temperature can only be observed at temperatures higher than 200 ºC. Due to reactor configuration, it is expected that higher water diffusional limitations will occur in the semi-continuous flow reactor, where the residue is packed inside the reactor. As temperature increases, and with it reaction rate, the accessibility to particles in the interior of the solid packed bed increases thus increasing reaction rate. This explains the non-linear behaviour of the effect of temperature in yield. In the batch reactor, mechanical agitation favours water contact with the solid where no packing effect exists.

3.3.4. Process yield in batch and semi-continuous systems

The process yield was calculated considering the solubilized material in the aqueous samples recovered during the experiment by equation 3.6.

$$Process\ Yield = \frac{WSC\ (g)}{100g\ RPS} \qquad \text{Equation (3.6)}$$

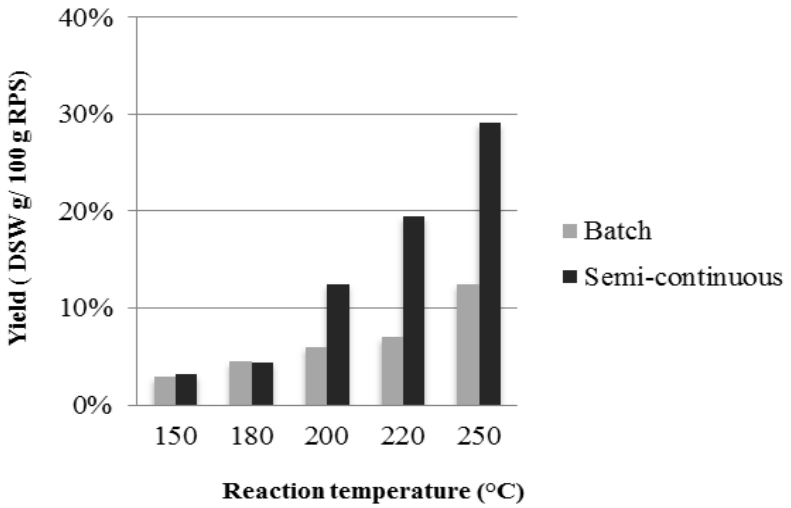


Figure 3.5: Batch and continuous process yields at 100 bar, 5 ml/min and 1h of reaction time.

Analysing figure 3.5, the highest yield was obtained for the experiments performed at 250 ºC in semi-continuous system achieving 29% of the total RPS mass converted into soluble compounds. At the same conditions, in the batch system the maximum yield obtained was lower than 20%. In terms of yield and especially at higher temperatures there is a significant difference between the two operating modes.

It is important to notice that after the dissolution of the polysaccharides in batch mode, this dissolved material remains in the reactor at the reaction conditions. Therefore, hydrolysis of soluble oligosaccharides and subsequent thermal degradation reactions will occur. In semi-continuous mode the resident time is much shorter (about 36 seconds) thus avoiding the thermal degradation of the dissolved polysaccharides. This could be the reason why, at the same reaction time, higher yields are obtained for the semi-continuous mode while a higher conversion was obtained for batch system.

3.3.5. RPS pre-treatment

In order to increase the surface area in the semi-continuous system, RPS was milled significantly reducing particle size (about 100 μm). By reducing particle size, mass transference would increase improving water diffusion and increasing hydrolysis reaction conversion rate. However, smaller particles would also increase packing inside the reactor.

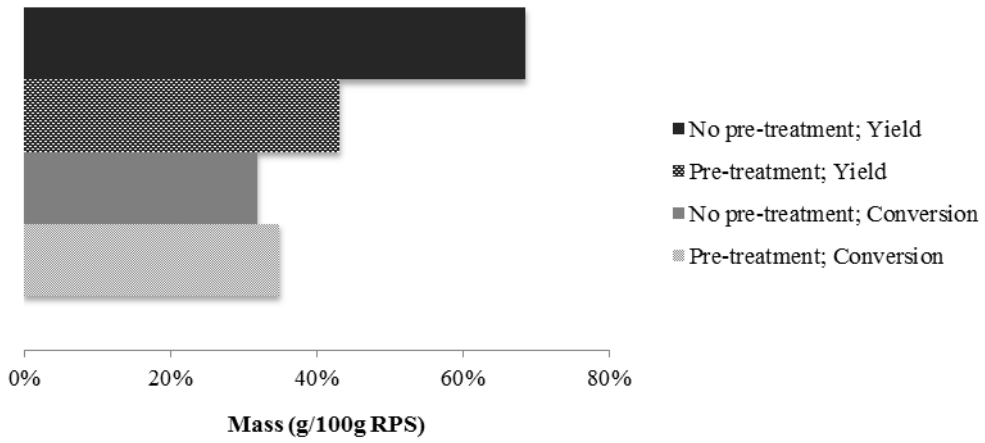


Figure 3.6: RPS pre-treatment operational advantages for the semi-continuous system operated at 250ºC.

Experiments using pre-treated RPS were only performed for the temperature of 250 ºC. Figure 3.6 compares both conversion and yield results achieved in the semi-continuous system operating at 250ºC when using the original raw material and the one that was previously milled. Considering the conversion, no significant differences were observed. However, considering the amount of soluble compounds recovered, it can be observed that the experiment performed with the milled RPS had a decrease of almost 50% (43%) when compared with the experiment performed with the original RPS (69%) (figure 3.7). By reducing the particle size, it would be expected that conversion would increase due to lower mass transfer limitation. Nevertheless this was not observed, which leads us to conclude that reactor design limitations were the major factor. The higher packing capability of smaller particles increases the compactibility of the packed bed decreasing the available sites for reaction, resulting in the same low conversion valus.

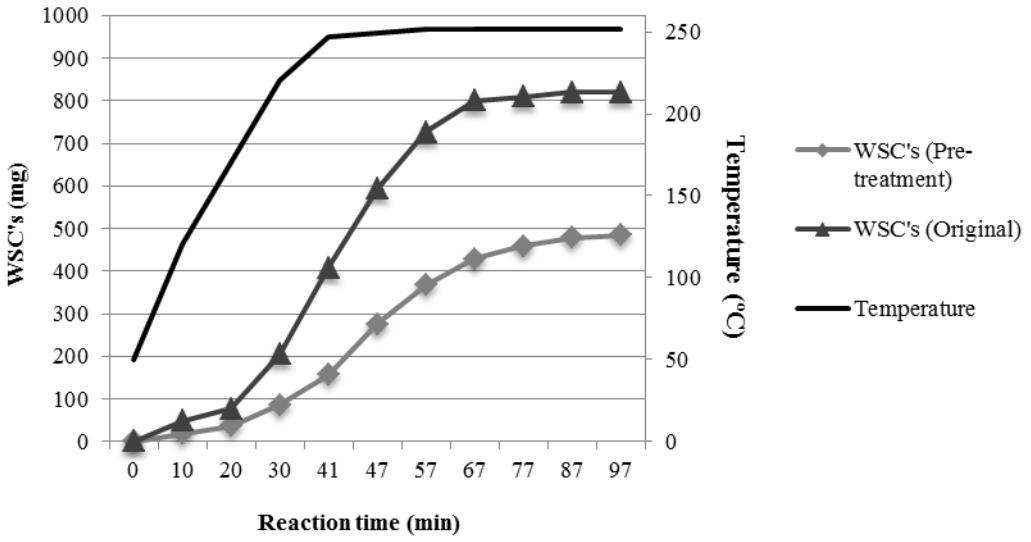


Figure 3.7: WSC's compounds and temperature profile using original and milled RPS.

Smaller particle size would not only improve hydrolysis reactions but could also promote a series of degradation reactions of glucose and xylose that can result in the formation of acids and volatile compounds that can be easily lost during the process without a proper collection system installation. This fact can justify the loss of the amount of soluble compounds collected when using the pre-treated raw material. Since the conversion achieved was not significantly high when compared with no-treatment application and due to the lower value achieved in terms of process yield, the pre-treatment of the RPS was considered as not benefic for process performance improvement.

3.3.6. Monosaccharide recovery and degradation compounds formation in batch and semi-continuous mode

Both systems performance was compared in order to evaluate which of them would allow higher monosaccharides recovery at the same time that degradation would be avoided. As already mentioned, 5-HMF is the major degradation product from hexoses and furfural is the major degradation product from pentoses (Fang, Z.; Xu, 2014).

Although batch system has the best results in terms of total process conversion, considering monosaccharides recovery, the semi-continuous process has a greater performance (Figure 3.8).

In figure 3.8A and 3.8B, it can be observed the proportion between xylose and glucose obtained at different temperatures for the semi-continuous and batch systems, respectively. Considering the semi-continuous process, significant amounts of glucose are only obtained at hydrolysis

performed at temperatures higher than 220ºC and, until this temperature, xylose was the predominant monosaccharide. Glucose presence drastically increases for the experiments performed at 250 ºC. Considering the batch system, monosaccharides recovery only starts to occur at temperatures higher than 180 ºC (Figure 3.8B) and in significantly low amounts.

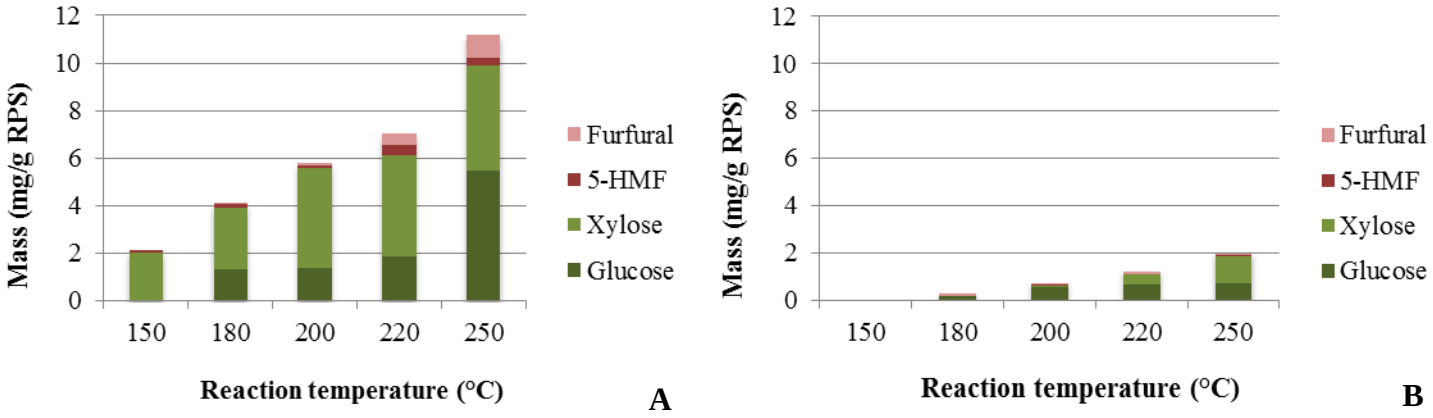


Figure 3.8: Monosaccharides recovery and degradation compounds formation in semi-continuous (A) and batch (B) systems per g of RPS treated.

Hemicellulose, due to its amorphous structure, is more easily hydrolysed than the crystalline structure of cellulose. On the other hand, at higher temperatures, the cellulose crystalline structure turns less rigid due to breakage of the long cellulose chains into smaller molecules, which will promote the release of glucose units (Wyman et al., 2005). This fact can easily justify the reason why xylose has the highest content in all experiments performed in the semi-continuous system until 220 ºC and at 250 ºC, the glucose amount increased due to cellulose structure breakage (Figure 3.8A).

Degradation reactions start to occur at 220 ºC for both systems (Figure 3.8). It is described in literature that the degradation reactions of five carbon sugars such as xylose are faster than the degradation reactions of six carbon sugars such as glucose (Dermibas, 2010). Analysing table 3.2, it can be observed that the amount of 5-HMF and furfural formed after 60 min of reaction time is slightly higher in the semi-continuous than for the batch system, especially when operating at higher temperatures. This is easily understood since they are formed from glucose (hexose) and xylose (pentose) degradation reactions.

Table 3.2: Degradation compounds formation in semi-continuous and batch systems.

	Total 5-HMF (mg)		*Total Furfural (mg)*	
T (°C)	***Semi-continuous***	***Batch***	***Semi-continuous***	***Batch***
150	0.15	0.00	0.00	0.40
180	0.35	0.12	0.18	0.65
200	0.26	0.35	0.25	0.63
220	1.17	0.44	1.31	1.03
250	1.03	0.65	3.57	0.31

Analysing both figure 3.8 and table 3.2, it is possible to conclude that, although in the semi-continuous system it was achieved the higher amounts in terms of degradation compounds, those reactions were slower than hydrolysis reactions since this system had also the highest amount of monosaccharides recovered. On the other hand, low amounts of degradation compounds were identified in batch system and at the same time only trace amounts of monosaccharides were also achieved. Considering that the conversion of RPS was higher in the batch system than when operating in the semi-continuous flow reactor (figure 3.4), these results suggest that the formation of volatile acids, which were not properly collected during sampling, occurred. Considering these results for both systems, the degradation compounds formation is not enough to justify the low monosaccharides recovery achieved in both systems knowing that 23,3% of the original raw material are polysaccharides as previously shown in table 3.1.

In table 3.3, it can be observed the increase of WSC when the operational temperature increases which allows the conclusion that cellulose and xylan structures are being hydrolysed by HCW, for both systems. However, only a percentage of WSC were experimentally identified as oligosaccharides and that percentage significantly decreases for higher temperatures. The decrease of the ratio oligosaccharides/WSC suggests that other structures are being hydrolysed and dissolved in water, for instance, lignin. This fact will be discussed further in this chapter.

Table 3.3: Oligosaccharide formation and monosaccharide recovery in semi-continuous process after 60 min of reaction, 100 bar and water flow rate of 5 ml/min.

T (°C)	*WSC (mg)*	*Oligosaccharides (mg)*	*% Total glucose recovered*	*% Total xylose recovered*	*Total initial mass of polysaccharides in RPS (mg)*
150	85	61	0,2	0.4	847
180	135	68	0.7	0.6	613
200	362	101	1.2	1.3	673
220	513	107	1.5	2.0	560
250	1060	159	3.8	3.1	628

* % Total glucose recovered: mg Glu/mg Cellulose in RPS

** % Total xylose recovered: mg Xyl/mg Xylan in RPS

Table 3.4: Oligosaccharides formation and monomers recovery in batch process after 60 min of reaction time and at 100 bar and 5 ml/min.

T (°C)	*WSC (mg)*	*Oligosaccharides (mg)*	*% Total glucose recovered*	*% Total xylose recovered*	*Total initial mass of polysaccharides in RPS (mg)*
150	294	59	0.0	0.0	2230
180	449	239	0.1	0.0	
200	600	238	0.3	0.2	
220	704	129	0.4	0.9	
250	1250	315	0.4	2.5	

The percentages of glucose and xylose recovered increase with temperature for all the experiments as can be observed also in table 3.3 and table 3.4. It can be also observed that performing HCW hydrolysis using the semi-continuous process at 250 ºC allows the highest percentage of monosaccharides recovery. The low amounts of monosaccharides recovered can suggest that a high percentage of glucose and xylose were degraded into 5-HMF and furfural, respectively, or even into volatile acids. Another hypothesis is that the reaction time was not high enough to fully convert polysaccharides into monosaccharides but rather into the

intermediary reactions product, which are soluble oligosaccharides. In order to develop this hypothesis, global mass balances were evaluated in order to determine what could have occurred to all the different compounds in the system.

3.3.7. Global mass balances

Global mass balances were performed for both systems in order to evaluate HCW hydrolysis process behaviour in detail considering all the components of RPS. Mass balances were performed following two approaches. A first global approach was performed according with direct experimental results such as initial and final HCW residue weigh, and WSC's amounts recovery after liquors were freeze dried. A second more detailed approach was performed to WSC's, evaluating its composition profile considering HPLC analysis of monomers and degradation compounds recovered. This analysis is important regarding the main goal of this study which is the evaluation of the potential of using HCW hydrolysis of RPS. By comparison of the results obtained from these two approaches, it was possible to perform a single component mass balance considering the global process.

Global hydrolysis process performance analysis: first approach

A global mass analysis of WSC's collected in both operational modes is represented in table 3.5.

At 150 ºC, in semi-continuous process, hydrolysis reaction extension is higher obtaining 72% of oligosaccharides in WSC's against 20% for the same temperature for the batch system. However, this behaviour is only observed again for the experiments performed at 250 ºC. For the temperatures of 180 ºC, 200 ºC and 220 ºC both systems had similar oligosaccharides percentages in WSC's. Considering batch system, the decrease of oligosaccharides content in WSC's does not follow a linear behaviour suggesting secondary reactions such as degradation reactions or recondensation. It can also be observed in table 3.5 a generally low oligosaccharide's yield when comparing batch with semi-continuous process at the same temperatures. Lignin and monosaccharides yields were also higher for the experiments performed in the semi-continuous system. Therefore, regarding the recovery of compounds of interest, the best option is the semi-continuous system operation.

Table 3.5: Characterization of WSC's obtained in semi-continuous and batch systems.

Semi-continuous system							
T (°C)	***WSC (mg)***	***Oligo in WSC (% w/w))***	***Lignin soluble in WSC (%w/w)***	***Mono in WSC (%w/w)***	***Others in WSC (% w/w)***	***Oligo yield (%w/w)***	***Mono Yield (%w/w)***
150	85	72	26	1.9	0.5	10	0.3
180	135	50	45	3.5	0.9	10	0.7
200	362	28	43	1.9	27.3	15	1.0
220	513	21	37	1.4	40.7	17	1.2
250	1060	15	31	2.9	51.2	19	3.7
Batch system							
T (°C)	***WSC (mg)***	***Oligo in WSC (% w/w))***	***Lignin soluble in WSC (%w/w)***	***Mono in WSC (%w/w)***	***Others in WSC (% w/w)***	***Oligo yield (%w/w)***	***Mono Yield (%w/w)***
150	294	20	17	0.0	62	3	0.0
180	449	53	11	0.4	35	10	0.1
200	600	40	19	1.1	40	10	0.3
220	704	18	10	1.5	70	6	0.5
250	1250	25	18	1.5	56	1	0.8

* Oligo: oligosaccharides.

** Mono: monosaccharides

*** Others: fat, proteins and ashes

**** Oligo Yield: $\frac{\text{the amount of oligosaccharides recovered}}{\text{maximum amount of polysaccahrides available (23,3\% of RPS)}}$

***** Mono Yield: $\frac{\text{the amount of monosaccharides recovered}}{\text{maximum amount of polysaccahrides available (23,3\% of RPS)}}$

****** Maximum polysaccharides represent the maximum amount that is possible to achieve after hydrolysis according to the initial RPS amount available. Corresponds to 23,3% of the initial raw material amount.

******* All the experiments were performed in duplicate and the average error for the semi-continuous system is 12% and the average error for the batch system is 18%.

An important aspect must be analysed further in detail. A significant amount of unknown compounds represented as "others in WSC" were identified in all experiments performed in both systems (Table 3.5). The percentage of unknown compounds increases with temperature achieving values higher than 50% for the experiments performed at 250 ºC. A detailed mass balance was performed as can be seen in Figures 3.9 and 3.10.

The mass balance takes into account the percentage of *"hydrolysed material"* representing the mass difference between the initial and final biomass in the reactor. The final residue that remained in the reactor after the hydrolysis reaction was weight and is represented as *"HCW residue".*

In both figures 3.9 and 3.10, it can be observed that the percentage of hydrolysed material increases with temperature at the same time that the percentage of HCW residue that still remains inside the reactor decreases, as expected. However, not all of the hydrolysed material was collected as WSC's, which means that the remaining percentage is representative of the *material loss* during the experiment. It can be observed that the material loss percentage decreases with temperature for both systems (Figure 3.9 and 3.10). For the semi-continuous system, that percentage starts at *ca.* 70% at 150 ºC achieving values lower than 10% at 250 ºC. For the batch system, the material loss is much higher and it is nearly 80% for all the temperatures tested. It is important to notice that, after sample collection, some deposit was observed in the liquors samples. That deposit could represent oligosaccharides structures that were hydrolysed during the experiment and solubilized at high temperatures but there are still too long to be solubilized in water at room temperatures. Additionally to these high carbon structures, during hydrolysis processing, repolymerisation of lignin structures can also occur justifying a lower amount of WSC's recovery (Brebu and Vasile, 2010). This situation was also observed in samples collected in semi-continuous system but with a less impact. However, it is also important to take into account that degradation reactions can also occur with the final formation of volatile acids that were not accounted.

Using the semi-continuous system at 250 ºC, 91% of the hydrolysed material was recovered as WSC's. It is interesting to notice that in the batch system it is not possible to achieve a percentage higher than 20% of the hydrolysed material in terms of WSC's. This can indicate a lower amount of water available to perform the hydrolysis comparing to the amount of water available in the semi-continuous system. Another hypothesis is the degradation reactions extension that in the batch system could be higher comparing with the semi-continuous system at the same temperatures.

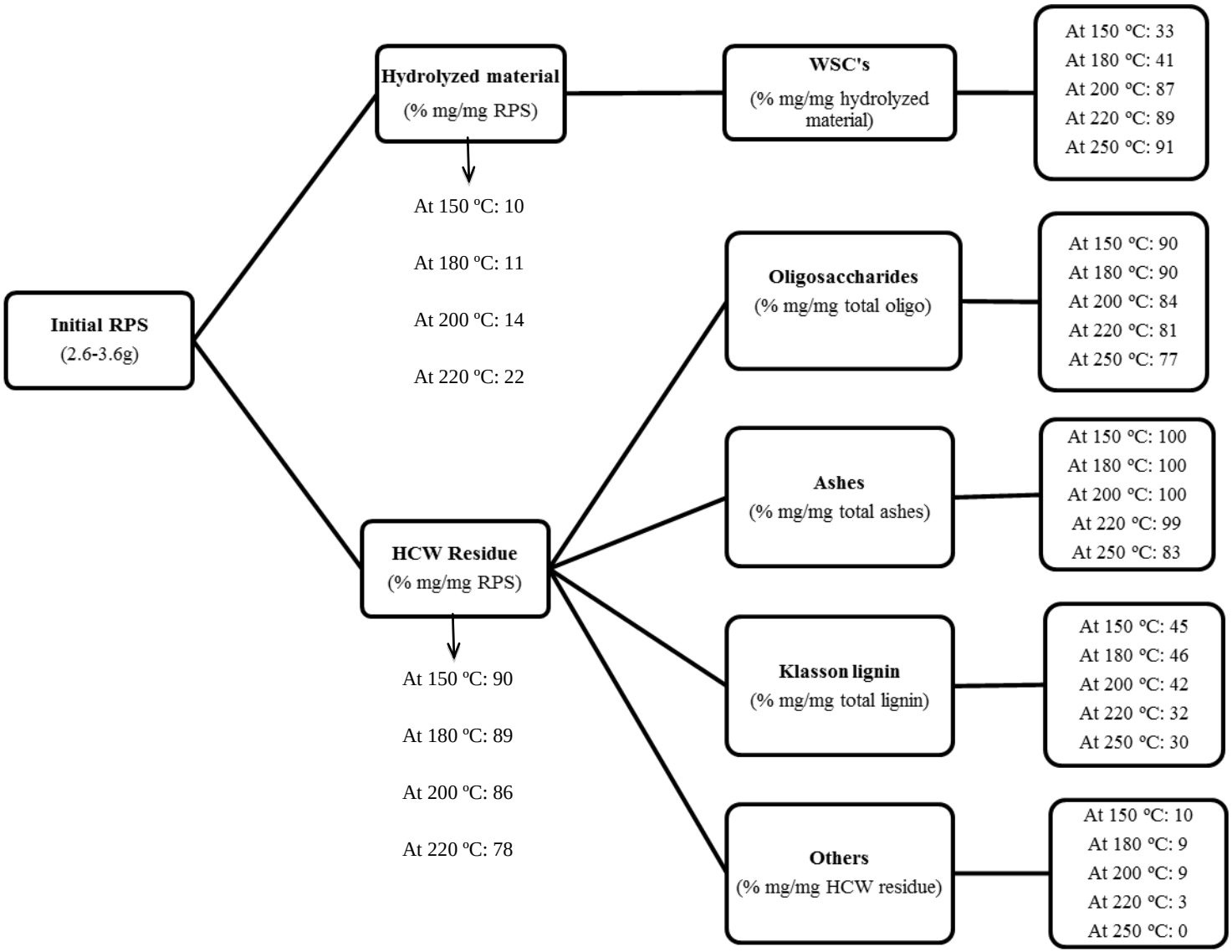


Figure 3.9: HCW hydrolysis in semi-continuous system at 100 bar and 5 ml/min: global mass balance diagram. Hydrolysed material corresponds to the mass that had disappeared during hydrolysis and it was obtained by the difference between initial mass of RPS inside the reactor and the mass of HCW residue weight after the experiment. WSC's mass was directly weight after liquors sample freeze-drying and the percentages corresponds to the amount of WSC's weight mass present in the amount of hydrolysed material obtained in the experiment. Material losses are the remaining percentage of WSC's not identified. Experiments average error is 12%.

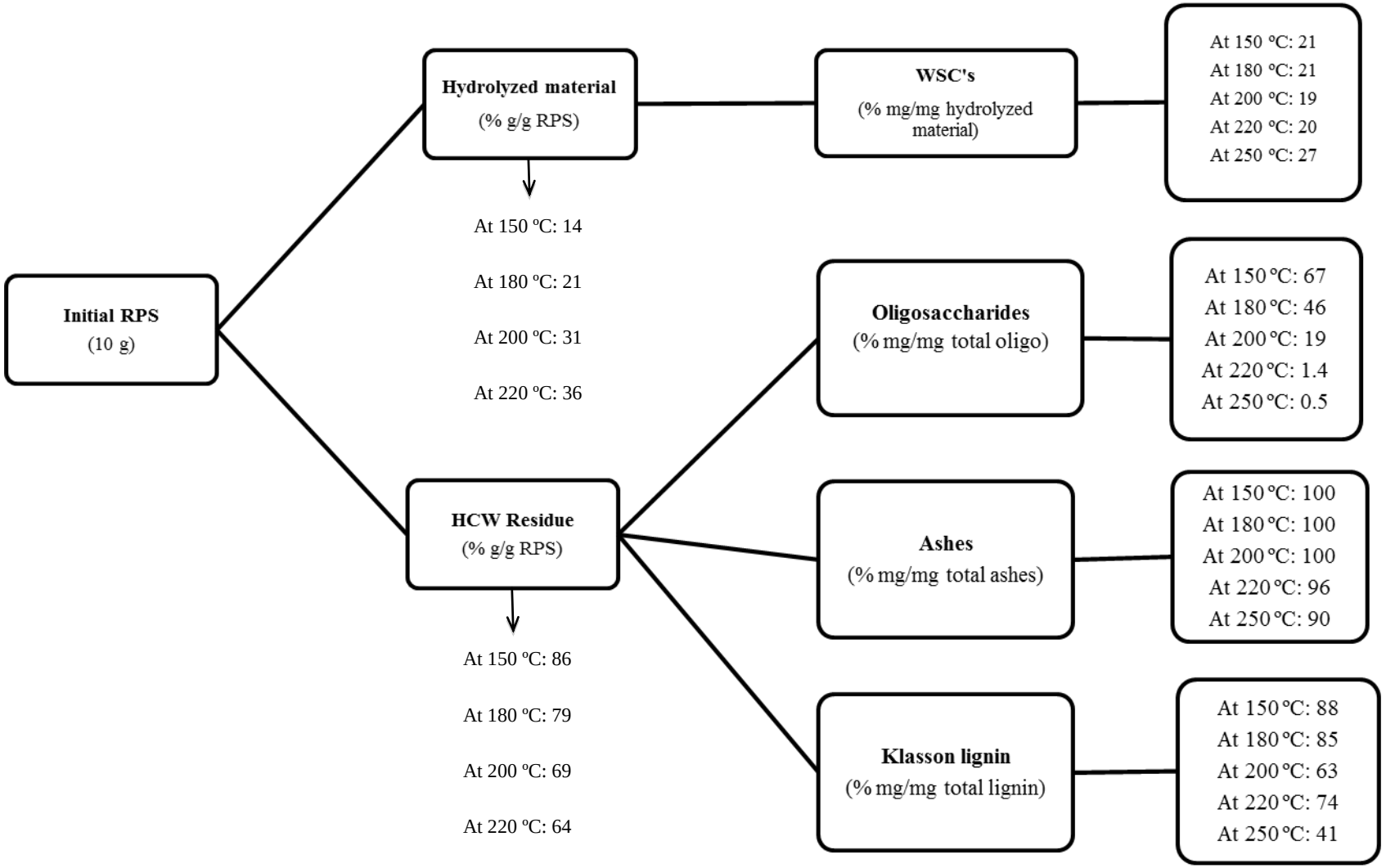


Figure 3.10: HCW hydrolysis in batch system at 100 bar: global mass balance diagram. Hydrolysed material corresponds to the mass that had disappeared during hydrolysis and it was obtained by the different between initial mass of RPS inside the reactor and the mass of HCW residue weight after the experiment. WSC's mass was directly weight after aqueous sample freeze-drying and the percentages corresponds to the amount of WSC's weight mass present in the amount of hydrolysed material obtained in the experiment. Material losses are the remaining percentage of WSC's not identified. Experiments average error is 18%.

In order to investigate the low WSC's recovered in some experiments, a detailed mass balance was also performed to HCW residue. HCW residue was studied in terms of oligosaccharides, ashes, Klasson lignin and other compounds final content (Figure 3.9 and 3.10).

In terms of oligosaccharides contents, it can be observed that in the semi-continuous system, higher percentages of oligosaccharides were identified in HCW residues (Figure 3.9) when compared with batch system. Regarding ashes mass balance for both systems, at temperatures lower than 200 ºC, no ashes were identified in WSC's, however at higher temperatures the partial solubilisation of ash start to occur (Figure 3.9 and 3.10). The solubilisation of ashes in HCW was already reported before (Brunner, 2009), (Ruiz et al., 2013). At 220 ºC and 250 ºC only about 90% of ashes total content was identified in HCW residues in both systems. In terms of lignin mass balance analysis, it can be observed that the amount of Klasson lignin present in HCW residue is higher in batch system that in semi-continuous (Figure 3.9 and 3.10). It is known that after lignin hydrolysis into its main building blocks, repolymerization reactions start to take place resulting in insoluble compounds (Brebu and Vasile, 2010). In batch, repolymerization of those structures could be favoured by the higher residence time when comparing with semi-continuous system where the residence time of lignin hydrolysed structures is much lower. In batch system, lignin hydrolysis into soluble compounds is only visible at 250ºC.

A small percentage of unknown compounds were also quantified in the HCW residue for the semi-continuous system that could represent lipids and fats. That percentage significantly decreases with temperature, being actually 0% at 250ºC. In batch system, no unknown compounds amount was identified in HCW residues (Figure 3.9 and 3.10).

WSC's detailed characterization and mass balance for both operational systems

In figures 3.11 and 3.13 it can be observed the mass profile of WSC's obtained for both systems at all the tested temperatures. Comparing WSC's profiles and the global mass balances presented previously in figure 3.9 and 3.10, it was possible to perform single mass balances for some of RPS components such as oligosaccharides, lignin and ashes. These results will be presented regarding each operational system used.

- **Semi-continuous system mass balances analysis**

A linear behaviour is observed for the experiments performed using the semi-continuous system. As observed in figure 3.11, the percentage of oligosaccharides, soluble lignin, monosaccharides and 5-HMF content in WSC's increase with temperature, as expected.

It is important to notice that, by using a semi-continuous system operating at 100 bar, 250 ºC and 5 ml/min, it was possible to recover, as solubilized compounds, nearly 50% of the initial carbon present in RPS, considering the amounts of oligosaccharides, monosaccharides and lignin identified in WSC's profile.

A total of nearly 20% of oligosaccharides was recovered which represents one of the components with major interest considering the goal of this study since they could be used directly by the microorganisms or be subject to a further hydrolysis step using enzymes. Moreover, 78% of total lignin was recovered as solubilized lignin which is a very important added value product that can be integrated in new and established chemical industry subsectors such as polymers, resins, adhesives, etc. (Stewart, 2008). These results demonstrates the potential of this process to hydrolyse lignocellulosic materials to obtain added value compounds such as smaller carbon structures which may be further used to growth microorganisms.

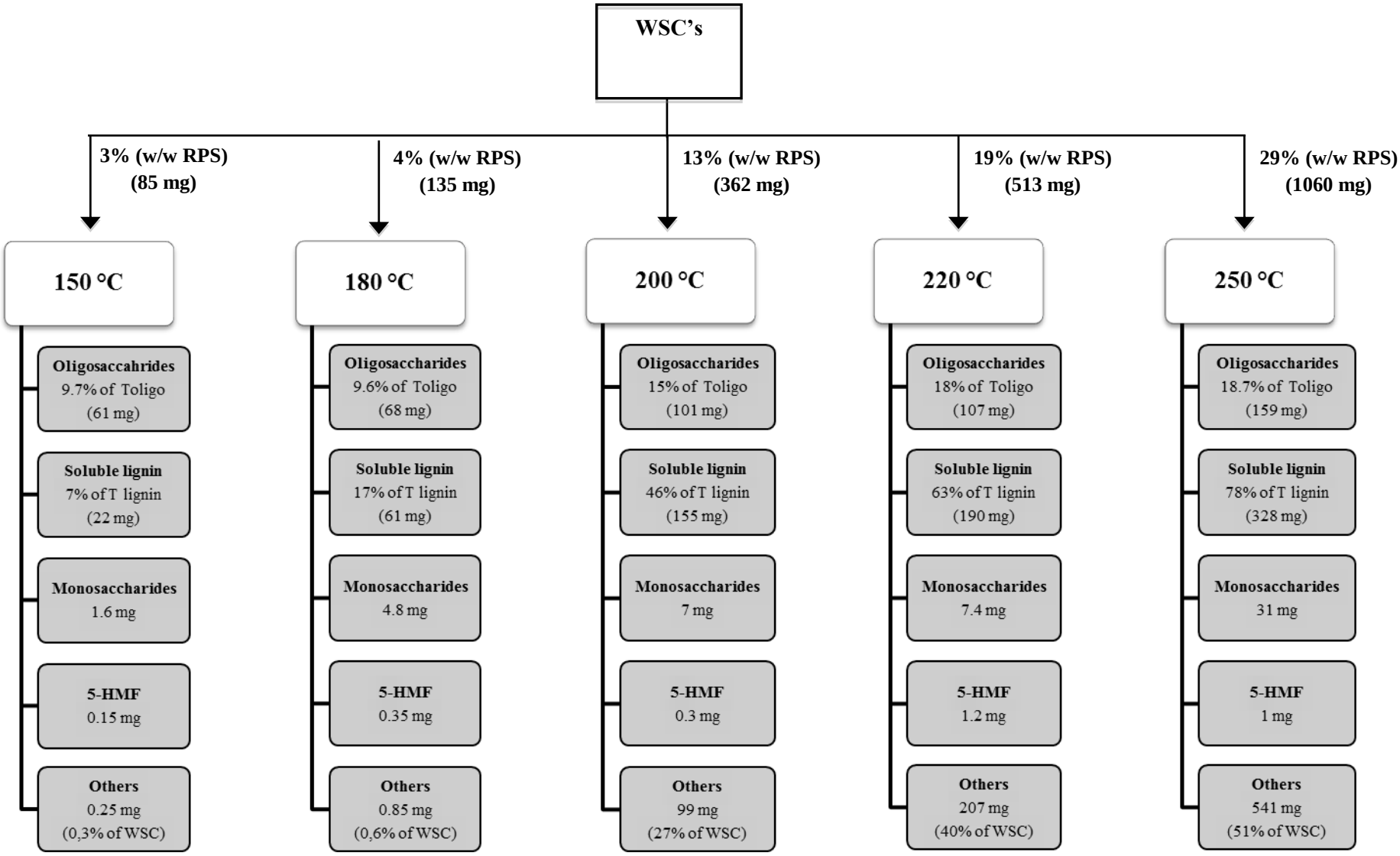


Figure 3.11: WSC's profile content from semi-continuous system experiments performed at 100 bar and 5 ml/min.

Analyzing together figure 3.9 (global mass balance) and 3.11 (WSC's profile), it can be observed that the total oligosaccharides losses (difference between the quantified amounts of oligosaccharides in HCW residue and solubilized in WSC's and the initial content in RPS) are between 0.3%-3.8%, increasing with temperature from 150ºC to 250ºC. At lower temperatures, this loss could be related with a weaker hydrolysis extension which results in longer carbon structures that are not solubilized in water at room temperature generating some deposit in the collected samples that was not taken into account. At higher temperatures, degradation reactions start to occur with a stronger influence.

Considering global lignin mass balance from figure 3.9 and the amount of soluble lignin detected in the liquors samples in figure 3.11, it can be observed that material losses decreases with the increase of temperature. The total lignin mass balance for the experiments performed in semi-continuous system is described in table 3.6.

Table 3.6: Lignin mass balance for the experiments performed at 100 bar and 5 ml/min in the semi-continuous system.

Mass (mg)	***150 ºC***	***180ºC***	***200 ºC***	***220 ºC***	***250 ºC***
Total initial lignin	312	353	335	305	422
Soluble lignin in WSC's	22	61	155	190	328
Klasson lignin	140	164	143	98	125
Lignin loss	150	128	37	17	0

*Lignin loss corresponds to the difference between the total initial lignin content and the amount of lignin detected as soluble lignin in WSC's and Klasson lignin.

Having into account that degradation of lignin into gas or solid carbon matter only occurs at very high temperatures that were not applied in this system, the 48% material loss at 150 ºC can be related with an incomplete hydrolysis which originates high molecular weight structures that were not solubilized in water at room temperature and precipitate. Also, lignin hydrolysis follows a two-stage mechanism starting with a very fast reaction, by breaking lignin–carbohydrate bonds, in which lower molecular weight and highly reactive lignin fragments are solubilized, followed by a slower reaction where the soluble fragments react between each other by recondensation and lignin repolymerization reactions (Ruiz et al., 2013). These second step can originate compounds which are no longer soluble in water at room temperature. At 250ºC, all the lignin content was distribute between the amount that was solubilized which corresponds

to 78% of the initial content and the remain percentage present in the HCW residue after RPS hydrolysis.

No significant differences between the amounts observed of Klasson lignin was detected for all the temperatures suggesting that the remaining lignin structure could require higher operational temperatures to be hydrolyzed into smaller units.

A significant percentage of unknown compounds, so called "others", was also verified in WSC's profile (figure 3.11). This percentage starts to be 0.3% at 150 ºC reaching 51% for the experiments performed at 250 ºC. These unknown compounds can be fat and proteins, which were not experimentally analysed in this study. Knowing that fat and protein initial content is 7.6%, the unknown compounds percentages found in all the experiments using the semi-continuous system are in conformation to what would be expected. Fats and oils are non-polar aliphatic compounds which solubility would increase at higher temperatures in which dielectric water constant is lower. On the other hand, protein hydrolysis only start to occur at temperatures higher than 230 ºC (Fangming Jin, 2014). It would then be expected that a weaker breakage of these structures would occur in the experiments performed at the lower temperatures, remaining then in HCW residue and at higher temperatures these compounds would then be accounted as solubilized compounds in WSC's collected. For the experiments performed until 200ºC, this amount is probably include in the unknown compounds content in HCW residue and for temperatures higher than 200ºC, fats and protein could start to be solubilized in aqueous solutions and accounted in the percentage of unknown compounds of WSC's.

However, the total amount of fat and proteins present in the original raw material does not justify the high percentages of unknown compounds in WSC's for the experiments performed at high temperatures. In figure 3.12, the total ashes mass balance for the experiments performed at 250 ºC in the semi-continuous system is presented. It can be observed that from a total of *ca.* 2 g of ashes present in the initial raw material, only 1.73 g was actually observed in the HCW residue present in the reactor after the experiment. Knowing this, it is possible to conclude that nearly 300 mg must have been dissolved and accounted as WSC's. From figure 3.12, 51% of the total 1060 mg of WSC's were accounted as unknown dissolved compounds (541 mg). Assuming that protein and fats contents representing *ca.* 200mg are accounted also in this amount, it is easily understood that the remain 300 mg not identified in the total ashes mass balance can also be present as WSC's, considering that now ashes amount would be expected to be accounted as lost material during the experiment. This fact it is according to what is described in literature about salts solubilization in sub- and supercritical water, as already mentioned before.

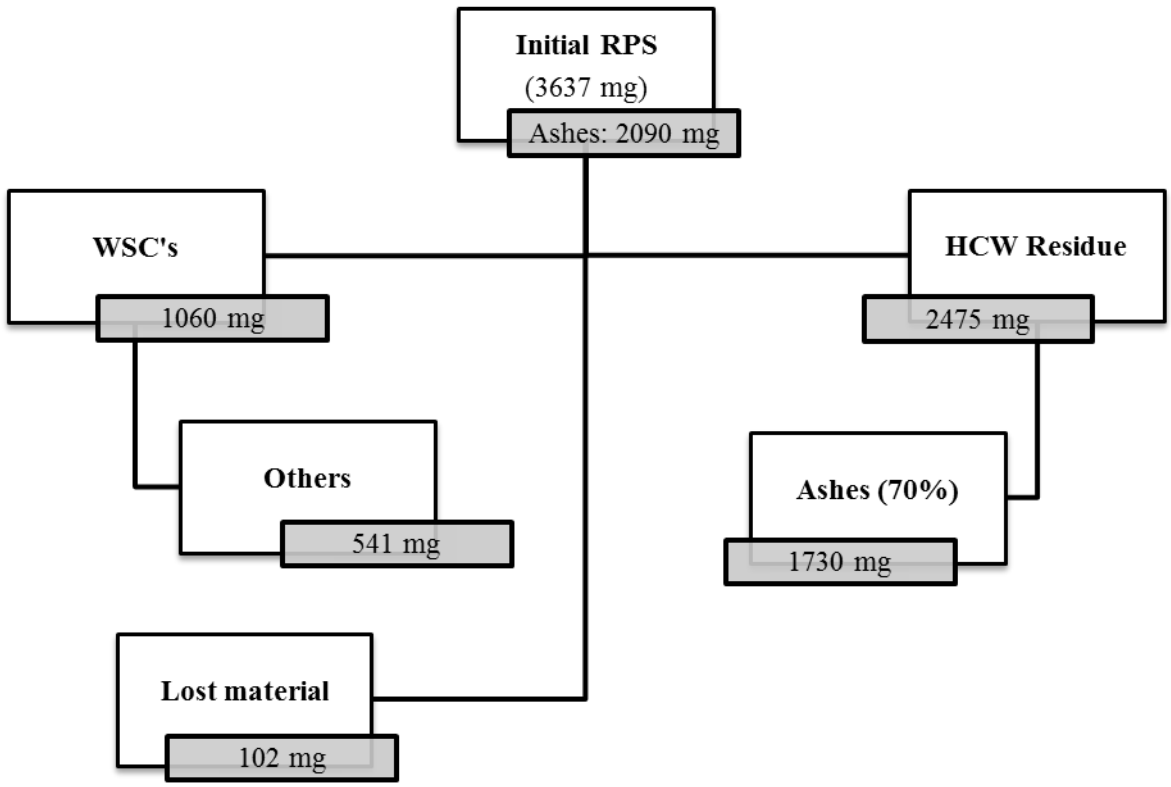


Figure 3.12: Ashes mass balance for the experiment performed at 250 ºC, 100 bar and 5 ml/min in the semi-continuous system.

The critical point it is an important mark in terms of water thermophysical proprieties. For instance, water solvent behaviour changes tremendously near the critical point as a function of temperature. As already mentioned, the solubility of some salts in HCW decreases drastically with temperature reaching almost zero at supercritical conditions (Brunner, 2009). However, there are some variations according with the type of salts in study. Salts such as NaCl are described as maintaining a relatively high solubility also at subcritical conditions (Toor et al., 2011). RPS metals composition profile was determined by atomic absorption spectroscopy (data not shown) in which it was concluded that calcium is by far the metal with the higher content, followed by magnesium, sodium, among others. It would be expected a similar behaviour for the salts containing this last metals in terms of HCW solubility capacity as it was verified for NaCl.

Batch system mass balance analysis

Analysing figure 3.13, a more irregular tendency can be observed considering the soluble oligosaccharides, soluble lignin and unknown compounds present in WSC's. Moreover, a general lower yield can be observed when comparing semi-continuous with batch operating system where significant lower amounts of products recovery can be observed (figure 3.11 e 3.13). Nevertheless, monosaccharides and 5-HMF amounts increase with temperature, as expected.

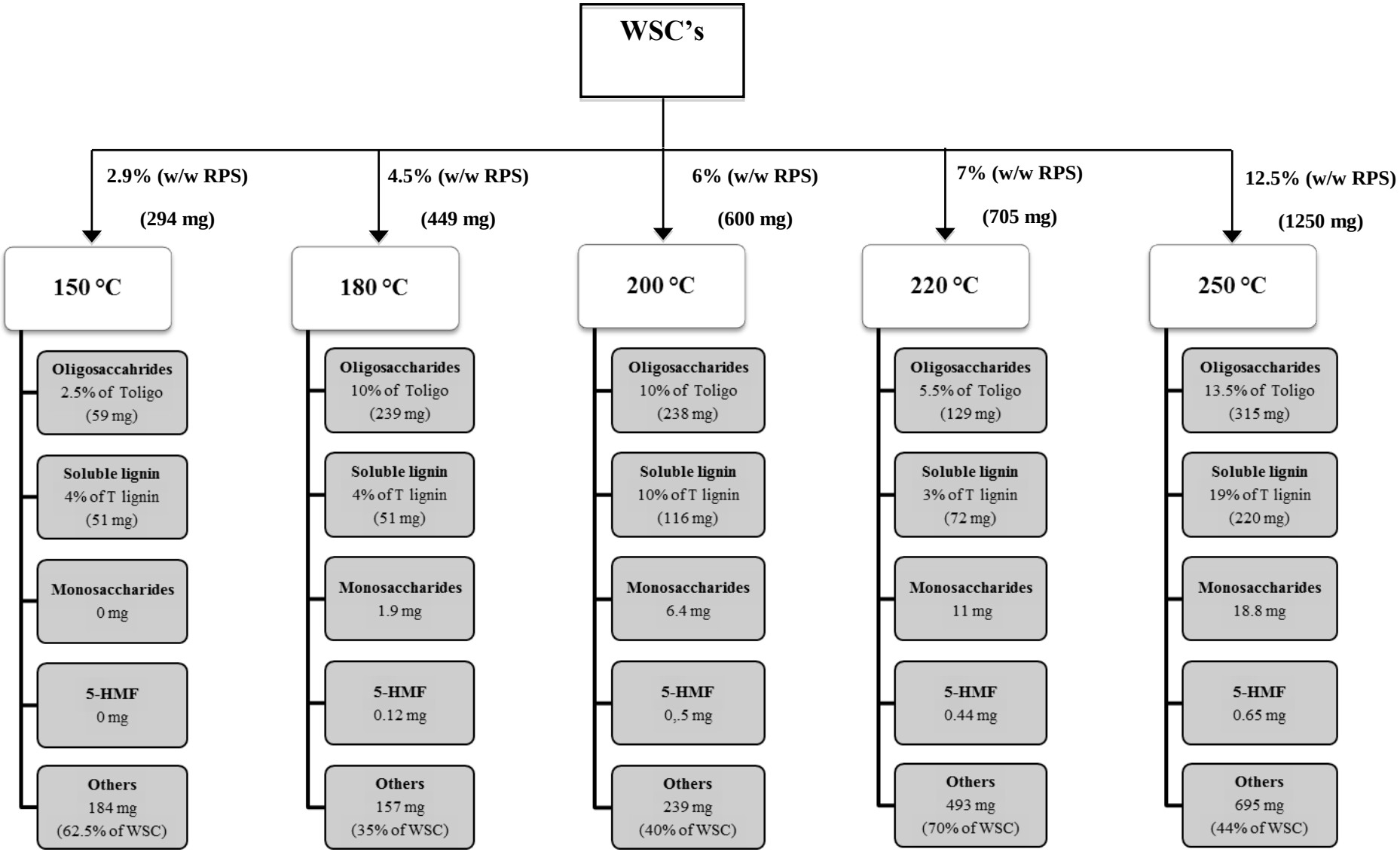


Figure 3.13: WSC's profile content from batch system experiments performed at 100 bar.

The oligosaccharide amount increases with temperature with the exception of 220 ºC where a sudden decrease is observe. This behaviour can be justified by the fact that xylan and cellulose structures are not hydrolysed at the same conditions. Xylan start to be hydrolysed at lower temperatures and the increase of oligosaccharides present in WSC's could be mainly due to xylan hydrolysis extension at the same time that cellulose structure is being weakened. At 220 ºC, considering batch operational mode, degradation reactions may begin to have a greater influence over hydrolysis, converting hemicellulose and decreasing the amount of oligosaccharides observed in WSC's after the experiment. Additionally, since cellulose hydrolysis extension increases with temperature, at 250ºC, a sudden increase in oligosaccharide content was again observed suggesting an increase derived from cellulose structure hydrolysis. This behaviour was not observed for the experiments performed at the same temperatures in the semi-continuous system may be due to the fact that in the semi-continuous system, the residence time is lower and hydrolysed oligosaccharides units from xylan and cellulose will be flowed out from the reactor avoiding further degradation when compared with batch system where higher residence time results in the formation of acids and volatile compounds not quantified.

Considering soluble lignin amount, the same behaviour is observed. As already mentioned for the total lignin mass balance in the semi-continuous system, a two-stage mechanism occurs during lignin structure hydrolysis. The second step is represented by recondensation and repolymerization reactions between the soluble fragments already hydrolysed from the main lignin structure during the first stage, resulting in the formation of high molecular weight units not soluble in water. At 220 ºC, temperature was not high enough to promote lignin hydrolysis in order to recover the main units but could promote the recondensation and repolymerization reactions between the soluble fragments hydrolysed at this temperature, justifying the sudden increase at this temperature. It is reported that lignin hydrolysis occurs at very high temperatures, therefore, at 250ºC, lignin structure hydrolysis extension should be higher resulting in higher amounts of soluble lignin present in WSC's.

The total lignin mass balance for the experiments performed in batch system is described in table 3.7.

Table 3.7: Lignin mass balance for the experiments performed at 100 bar and 5 ml/min in batch system.

Mass (mg)	*150 ºC*	*180ºC*	*200 ºC*	*220 ºC*	*250 ºC*
Initial RPS			10000		
Total initial lignin			1160		
Soluble lignin in WSC's	51	51	116	72	220
Klasson lignin	1021	986	731	864	476
Lignin loss	88	123	313	224	464

It can be observed in table 3.7 an increase of lignin loss with temperature. Although the amount of Klasson lignin decrease with temperature, the corresponding difference is not then identified as soluble lignin suggesting that some degradation or chemical transformation is occurring to the fragments that were hydrolysed (according with the verified decrease amount of Klasson lignin) but not in a sufficient extension to turn them soluble into water. Higher temperatures and/or higher reaction times should be tested to evaluate its influence in terms of lignin recovery.

As also mentioned for the semi-continuous system, a large amount of unknown compounds was also verified in WSC's profile for batch system (figure 3.13). Although the percentages of unknown compounds present in WSC's collected denotes an irregular behaviour when increasing temperature, it is important to notice that in mass terms, the amount of unknown compounds is increasing with temperature. As already mentioned before, a weak hydrolysis extension can promote the deposit formation after liquors collection justifying the increase verified of unknown compounds amount for the experiments performed until 200ºC. For the experimental temperatures of 220ºC and 250ºC, other parameters could be involved. Analysing figure 3.10, it is possible to conclude that no ashes are supposed to be present in this amount since 100% of the total ashes was quantified in HCW residue for the experiments performed at 150ºC, 180ºC and 200ºC. On the other hand, for the experiments performed at 220ºC and 250ºC, a material lost of 4% and 10% of the total initial ashes mass was verified. Considering that the initial RPS in batch system was 10000 mg, the total initial amount of ashes was 5650 mg that should be distribute between HCW residue and WSC's since it was already proven that some ashes dissolution can occurred. The significant increase of unknown compounds in WSC's profile for batch system for the experiments performed at the higher temperatures can be related with fat and proteins hydrolysis and dissolution that can occur at higher temperatures and also due to some ashes content solubilization in HCW during experiment.

3.3.8. Dynamic mass balance

In this section, HCW hydrolysis dynamic behaviour will be discussed. The evaluation of a mass balance that describes the behaviour of this kind of system along the reaction time is very important in order to assess its viability in industrial and economical terms. An accurate knowledge of process development according to operational temperature and reaction time pre-selected would reduce operational cost and improve process efficiency. Considering this, added value by-products production along time for all the temperatures in study was evaluated to discuss which conditions would turn HCW hydrolysis process more profitable.

3.3.8.1. Products production along time

The main compounds of interest in this process are WSC's, oligosaccharides, monosaccharides and lignin. In each experiment, liquors samples were taken after the desired temperature was reached, corresponding to time 0. The time taken to heat the reactor to that temperature was discarded. After reaching the desired operational temperature, samples were collected every 10 min until 30 min of reaction time, collecting in a single sample the last 30 min until a final 60 min of reaction time.

In table 3.8 is represented WSC's obtained during time for all the temperatures in study for both operational modes. WSC's recovery increases with time for all the temperatures for both systems. It is important to notice that WSC's increase between reaction times is more evident for temperatures higher than 200 ºC where an increase higher than 30 mg/g residue can be observed each 10 min.

For the operational temperatures of 150 ºC and 180 ºC, no significant differences are observed comparing batch and semi-continuous systems at the same reaction times. However, for the experiments performed at temperatures and reactions times higher than 200 ºC and 20 min, higher amounts of WSC's were recovered in the semi-continuous mode than in batch. The highest recovery of WSC's was obtained for the experiments performed in the semi-continuous system during 60 min at 250ºC. However, it is important to evaluate the profile of this WSC's collected since not all would be the target products.

Table 3.8: WSC's obtained during 60 min of reaction time for all the temperatures in study. Experiments performed at 100 bar.

WSC's (mg/g residue)					
Semi-continuous process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	9	16	48	79	120
0→10 min	14	21	83	111	171
10→20 min	17	30	100	132	208
20→30 min	23	33	110	150	239
30→60 min	32	44	125	169	291
Batch process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	9	22	29	42	52
0→10 min	20	32	55	57	70
10→20 min	26	36	57	60	90
20→30 min	23	39	55	58	97
30→60 min	29	45	60	70	125

In table 3.9, 3.10 and 3.11, it is possible to evaluate oligosaccharides, glucose and xylose recovery for all the conditions in study. Oligosaccharides, glucose and xylose recovery increases with reaction time and temperature in most of the conditions evaluated but not with such an evident behaviour.

Table 3.9: Oligosaccharides obtained during 60 min of reaction time for all the temperatures in study. Experiments performed at 100 bar.

Oligosaccharides (mg/g residue)					
Semi-continuous process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	6	9	12	21	20
0→10 min	11	12	22	27	27
10→20 min	15	15	25	30	31
20→30 min	18	17	29	33	36
30→60 min	23	22	35	41	44
Batch process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	3	8	12	22	21
0→10 min	6	10	24	22	17
10→20 min	10	15	24	24	23
20→30 min	4	21	26	17	28
30→60 min	6	24	24	13	32

In the batch system, oligosaccharides recovery decreases at 220 ºC, for the experiments performed with reaction times higher than 20 min suggesting that degradation reaction would be faster than hydrolysis rate. The development of an energetic cost balance would be necessary to evaluate the benefit of increasing the operational temperature to 250ºC and reaction time to 60 min in order to recovery more *ca*. 8 mg/g residue of oligosaccharides (from 24 mg/g residue at 220ºC during 20 min to 32 mg/g residue at 250ºC during 60 min)(table 3.9). Moreover, for the semi-continuous system no significant increase was observe considering the experiments performed at 220ºC and 250ºC. This increase would be economically benefit if the monosaccharides content in the experiment performed at 250ºC would be higher than for the experiment performed at 220ºC. It can be observed in table 3.10 that it is.

In table 3.10 is evident the increase of glucose recovery with temperature and reaction time. Considering both systems, the semi-continuous mode allows the recovery of glucose at lower temperature. In batch significant amounts of monosaccharides were only obtained at a temperature higher than 220ºC and 20 min.

Table 3.10: Glucose obtained during 60 min of reaction time for all the temperatures in study. Experiments performed at 100 bar.

Glucose (mg/g residue)					
Semi-continuous process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	0.1	0.5	0.4	0.6	2.4
0→10 min	0.2	0.7	0.6	0.7	3.0
10→20 min	0.3	0.9	0.8	0.9	4.8
20→30 min	0.4	1.0	1.1	1.0	5.3
30→60 min	0.5	1.4	1.5	1.8	5.5
Batch process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	0.0	0.0	0.0	0.0	0.1
0→10 min	0.0	0.0	0.0	0.0	0.3
10→20 min	0.0	0.0	0.1	0.2	0.4
20→30 min	0.0	0.0	0.3	0.6	0.8
30→60 min	0.0	0.2	0.5	0.7	0.7

Xylose recovery follows the same behaviour observed for glucose production (table 3.11). It is clearly evident that it is not possible to recover any monosaccharide in the batch system if operational temperature is lower than 220ºC and reaction times lower than 20 min. Experiments performed in batch at 250ºC and reaction times higher than 20 min will actually promote xylose degradation (table 3.11). The higher recovery was achieved in the experiments performed at 250ºC in the semi-continuous system but low improvements were observed in the last 30 minutes of reaction suggesting that no benefits would be observed considering the operational costs necessary to run the system for more 30 min (table 3.10 and 3.11). An accurate energy balance development would be important to support the operational conditions selection but analysing all the results, in terms of oligosaccharides and monosaccharides recovery, the semi-continuous system has proven to be the most efficient system operating at 250ºC and with a reaction time of at least of 20 min.

Table 3.11: Xylose obtained during 60 min of reaction time for all the temperatures in study. Experiments performed at 100 bar.

Xylose (mg/g residue)					
Semi-continuous process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	0.1	0.1	0.3	0.4	0.3
0→10 min	0.2	0.2	0.3	0.5	0.7
10→20 min	0.2	0.2	0.4	0.5	0.9
20→30 min	0.3	0.2	0.4	0.5	1.0
30→60 min	0.4	0.3	0.6	0.5	1.1
Batch process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	0.0	0.0	0.0	0.0	0.3
0→10 min	0.0	0.0	0.0	0.0	0.4
10→20 min	0.0	0.0	0.0	0.1	0.2
20→30 min	0.0	0.0	0.0	0.1	0.1
30→60 min	0.0	0.0	0.1	0.2	0.1

Table 3.12: Soluble lignin obtained during 60 min of reaction time for all the temperatures in study. Experiments performed at 100 bar.

Soluble lignin (mg/g residue)					
Semi-continuous process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	1	5	18	34	38
0→10 min	3	10	36	54	61
10→20 min	5	13	43	61	71
20→30 min	6	15	48	65	79
30→60 min	8	20	54	72	90
Batch process					
Time (min)	***150 ºC***	***180ºC***	***200ºC***	***220ºC***	***250ºC***
0	2	3	7	4	14
0→10 min	2	7	9	9	14
10→20 min	3	7	17	13	19
20→30 min	5	5	11	11	16
30→60 min	5	5	12	7	22

Soluble lignin recovery can be observed in table 3.12. It can be easily observed that batch system allow a very low recovery of soluble lignin taken expression only for the experiments performed at 250ºC. It is also interesting to notice that the maximum amount of soluble lignin recovered do not correspond always to 60 min of reaction time. For instance, for the experiments performed at 200ºC and 220ºC, the maximum recovery of soluble lignin is achieved at 20 min of reaction suggesting that, prolonging the reaction, would promote de degradation or chemical transformation of the hydrolysed lignin fragments that were already solubilized. Considering all the experimental conditions, the highest recovery amount was achieved for both systems at 250ºC but the lignin amounts recovered in the semi-continuous system were largely higher when compared with batch mode. At 250ºC, the amount of soluble lignin obtained during the heating time is higher than the amount recovery after 60 min of reaction in the batch mode. The highest amount of soluble lignin is obtained when treating RPS in the semi-continuous mode operating at 250ºC and 60 min.

3.3.8.2. Severity factor

In table 3.13, the severity factor was calculated considering operational conditions in terms of temperature and reaction time used in this study. pH influence was also considered in the calculation of the severity factor only for the experiments performed with 60 min of reaction time.

Table 3.13: Severity factor calculations for the operational conditions in study for both operational systems.

Severity factor (log R_0)					
t (min)	***Temperature (ºC)***				
	150	**180**	**200**	**220**	**250**
10	2.5	3.4	3.9	4.5	5.4
20	2.8	3.7	4.2	4.8	5.7
30	2.9	3.8	4.4	5.0	5.9
60	3.3	4.1	4.7	5.3	6.2
60 (log R_0')	4.0	4.9	5.4	6.4	7.4

Analysing table 3.13, it can be observed that higher the temperature or higher the residence time, higher the severity factor, as expected. It is important to emphasize that, ideally, operational conditions should be "severe" enough to promote major structures hydrolysis but not strong enough to extent hydrolysis to the degradation mechanisms. For the experiments performed at 150 ºC and 180 ºC, the severity factor increases regularly with time but the severity factor obtained for the experiment performed at 200 ºC during 10 or 20 min is very similar to the severity factor obtained for the conditions at 180 ºC and 60 min. Considering the combined severity factor that takes into account pH influence, it can be observed in table 3.13 that the severity factors are considerably higher when compared with the calculation that has not have into account pH influence. If it is considered the pH value of the reaction medium as a parameter to control during experiment, a higher severity factor should be taken into account to select the best operational conditions. Nevertheless, the same tendency was observed. Higher the temperature, higher the severity factor for the same reaction time of 60 min.

In order to have an accurate performance analysis, the severity factor must be combined with at least one process parameter that represents hydrolysis performance for instance the amount of WSC's obtained at each applied condition. In figure 3.14, it is possible to observe WSC's recovered as a function of the severity factor for both systems.

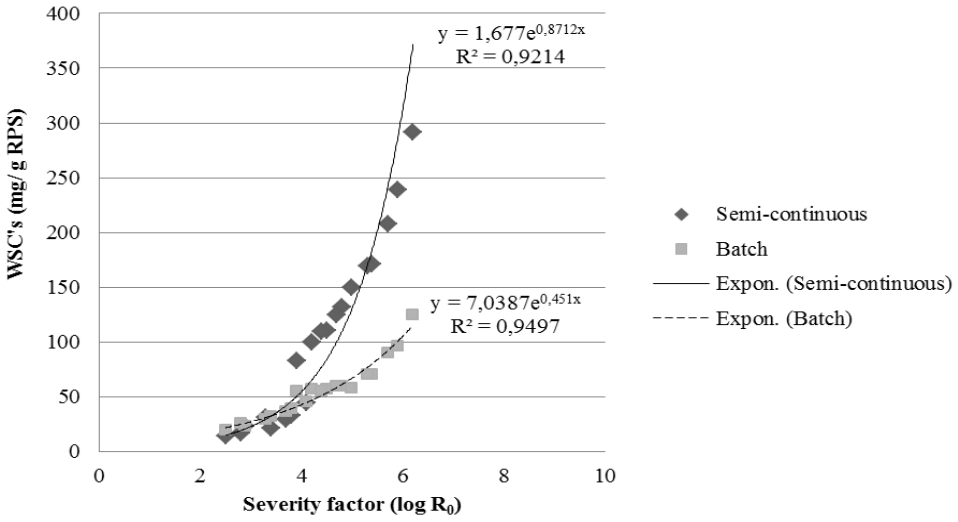


Figure 3.14: Severity factor as a function of WSC's recovery in both operational modes.

Analysing figure 3.14, it is possible to observe that until a severity factor of about four, a low amount of WSC's will be recovered for both systems. At higher severity factors, WSC's recovery follows an exponential behaviour, as represented in figure 3.14 with a high correlation factor for both systems. Therefore, it is possible to conclude that, for a high WSC's recovery, operational conditions which correspond to a severity factor higher than four should be applied. By direct observation of table 3.13, this indicates that temperatures above 200 ºC should be selected. Reaction time depends on the temperature applied since the higher the temperature, a lower reaction time is necessary to maintain the severity factor at values higher than four.

It is interesting to notice the difference between both systems response according with the severity factor combined with the WSC's recovery. In batch system, WSC's recovery also follows an exponential behaviour but with a lower exponent parameter. This means that process parameter have a higher influence in WSC recovery in the semi-continuous system than in batch system, in other words, the severity of the process has a higher influence in the semi-continuous process. Moreover, in the semi-continuous system higher a WSC recovery is achieved at milder conditions than for the batch system.

3.4. Conclusions

RPS is generated in large amounts by wastewater treatment facilities of recycled paper plants, representing a serious disposal problem. HCW has the potential to be used as a solvent and a reactant for RPS hydrolysis due to its unique chemical and physical characteristics. In this chapter it was evaluated the potential of RPS as an alternative carbon source for microorganisms growth by using hot compressed water hydrolysis to convert polysaccharides structures, such as, cellulose and hemicellulose, into their monosaccharides components. Two operations modes for RPS hydrolysis using HCW were compared: semi-continuous and batch mode. The performance of both systems were analyzed in terms of conversions, product yield and degradation product formation. Although in batch mode it was possible to achieve higher RPS conversion, in the semi-continuous systems higher yields of WSC's and monosaccharides were achieved. By using a semi-continuous system at 100 bar, 250 ºC and a water flow rate of 5 ml/min, it was possible to nearly 50% of the initial carbon present in RPS, considering the soluble amounts of oligosaccharides, monosaccharides and lignin identified in WSC's recovered. Detailed mass balances were performed for both operational modes with strong evidences that degradation reactions extension was higher in the batch mode. Mass balances along the reaction time were also performed for both systems. Although an accurate energy balance development would be important to support the operational conditions selection, analysing all the results, in terms of oligosaccharides and monosaccharides recovery, the semi-continuous system has proven to be the most efficient system operating at 250 ºC and with a reaction time of at least of 20 min.

An analysis of the severity factor was performed for both operational modes. In the semi-continuous mode WSC's recovery follows an exponential behaviour at severity factors higher than four.

4

Grape pomace valorization through HCW treatment

4.1

Grape pomace phenolic compounds extraction using HCW

4.1.1 Introduction

Fruits, vegetables, spices and aromatic herbs are known as rich natural sources of phenolic compounds. In nature, the main classes of these phytochemicals are flavonoids and phenolic acids in free or complex forms. Polyphenols are considered by numerous studies to have several biological effects towards human health such as antioxidant and antimicrobial activities (Aliakbarian et al., 2012). It has been reported by several researchers that the antioxidant activity of the phenolic compounds show a high correlation with the prevention of some prevalent chronic diseases such as coronary heart disease and cancer. Some studies have also proposed that these compounds could have an important role in the ageing process (Barth et al., 2005; Candrawinata et al., 2015; Galluzzo and Marino, 2006). These benefit effect in human health is considered to be related with an important role in regulating the imbalance between oxidative stress and antioxidants in the body (Candrawinata et al., 2015; Liang et al., 2014; Scalbert et al., 2005)

Wine waste, mainly skins, seeds and stems, represents 20% of the processed grape weight and are considered as added value resources due to their high phenolic compounds content (Rajha et al., 2014). These by-products obtained after wine production constitute a cheap source for extraction of phenolic compounds, mainly flavonoids, which can be used as food supplements or in the production of phytochemicals (González-Paramás et al., 2004). Most phenolic compounds found in wine can act as antioxidants (Rajha et al., 2014). Moreover, wine production waste products are also characterized by high contents of phenolic compounds due to an incomplete extraction during wine production. It have been reported that anthocyanins can be extracted from grapes skin and procyanidins from the seeds (Ivan et al., 2011).

In recent years, the interest in extracting phenolic compounds from GP has been increasing and several studies have shown that phenolic compounds from GP can be extracted using several types of organic solvents, mainly methanol, acetone or ethanol (Candrawinata et al., 2015; Fontana et al., 2013). However, the use of organic solvents is not practical in industry mainly due to downstream and environmental problems. Water has been studied as a suitable alternative extraction solvent due to the fact that not only has the ability to extract the phenolics but also it is a cheap, non-toxic and easily accessible solvent (Rajha et al., 2013); (Reis et al., 2012).

In this section, phenolic compounds extraction process from GP was studied using organic solvents to evaluate GP phenolic content potential and HCW as the alternative environment friendly extraction solvent.

4.1.2 Materials and Methods

All the methods used in this section were described in the Chapter 2: Materials and Methods. A brief review is presented below. All the analysis described were performed to the liquors collected after HCW extractions at different temperatures, 5 ml/min and 100 bar as well as to the extracts obtained from the conventional extraction methodologies applied.

Total Phenolic Content (TPC) was determined by the Folin-Ciocalteau colorimetric method (Folin and Ciocalteau, 1927). Protein precipitation was performed using 800 µl of each hydrolysates sample and adding 120 µl of Trichloroacetic Acid (Scharlau 99.5%). Samples were stored at -20 ºC for 5 min, then at 4 ºC for 15 min and centrifuge (Heraeus sepatech, Biofuge 13 Centrifuge) for 15 min at 12 000 rpm. To 20 µl of resulted supernatant, it was added 1.58 ml of Mili-Q water, 100 µl of Folin-Ciocalteu reagent (MERK) and the resulted solutions were stored at room temperature for 8 min. Then, it was added 300 µl Sodium Carbonate (Sigma) solution (200 g/L) and incubated in water bath at 40 ºC for 30 min. The absorbance was measured at 750 nm using a spectrophotometer (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA).

The identification and quantification of individual phenolic compounds present GP extracts was performed using a Thermo Scientific Surveyor Plus HPLC System with a UV detector and BDS HYPERSIL C18 column (length 250mm; I.D. 4mm) at a constant temperature of 40 ºC (Schieber et al., 2001). The mobile phase was a mixture of two eluents. Eluent A was a 2% (v/v) solution of glacial acetic acid in ultrapure water, and eluent B was a 0.5% (v/v) solution of glacial acetic acid in a 1:1 (v/v) mixture of ultrapure water and acetonitrile. The gradient program was: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min). Calibration curves were built with ten solutions of either furfural or 5-HMF, with concentrations ranging from 25 to 250 ppm.

Three extraction solvents mixtures were used to perform the phenolics extraction from grape pomace in order to evaluate the raw material initial content: ethanol, acetone and citric acid. The extractions methods are described in Chapter 2: Materials and Methods.

4.1.3. Results and discussion

The main challenge is to develop an efficient methodology capable to recover phenolic compounds in its original structure and with original proprieties. Several analytical methods have been developed for the total phenolic content determination at the same time that chromatographic and spectrophotometric analysis are continuously improved in order to achieve adequate separation of phenolic molecules, their subsequent identification and quantification. Considering the phenolics profile of GP described in the literature, and the available standards, six phenolics compounds were selected for HPLC analysis: gallic acid, caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid and resveratrol. All these compounds are considered non-flavonoids. Gallic acid is a hydroxybenzoic acid (C6-C1 structure); p-coumaric acid, caffeic acid and ferulic acid are hydroxycinnamic acids (C6-C3 structure); chlorogenic acid is the ester of caffeic acid and resveratrol is a stilbenoid. All these phenolic compounds have an important role in pharmaceutical and food industry as natural antioxidants or as ingredients of functional food (Cabrita et al., 2012). These 6 phenolic compounds were analyzed in each sample collected during HCW treatment as well as in the extracts obtained from organic extractions performed using hydroalcoholic solution of water and ethanol (75:25 v/v), water and acetone (20:80 v/v) and a solution of acid citric (3 g/L).

4.1.3.1. Total phenolic compounds in red GP

The method mostly used for total phenolic quantification is the Folin-Ciocalteau assay which acts on the phenols due to their reductive properties. The method is an electron transfer based assay and measures the reducing capacity to estimate total phenolic content of biological materials. Gallic acid is used as standard compound and TPC is given as gallic acid equivalents (GAE). In detail, it consists in the reduction of phosphomolybdic acid to a blue colored complex by phenolic compounds in alkaline conditions. However, this method is not specific since some phenolic groups are found in extractable proteins or reducing substances such as ascorbic acid can also participate in the reduction reaction (Lorrain et al., 2013).

Several parameters during wine production process can affect significantly the final phenolic composition in wine and consequently in the remaining residues, such us the type of extraction and contact time, heat and enzymatic treatments applied during the process (Frankel et al., 1998). It was reported that high temperatures used during extraction or storage promotes the degradation of anthocyanins and, consequently, a decrease in the color and total phenolics content (Burin et al., 2010).

A wide broad range of TPC was already reported for several red grapes varieties and by-products using different solvent mixtures including methanol, ethanol or petroleum ether. Those content can range from 5.4-14.3 mg GAE/g using a mixture of MeOH/H_2O/HCl (90:9.5:0.5 v/v)(Anastasiadi et al., 2012), 32.6-74.7 mg GAE/g using acidified methanol (0.1% HCl) (Ivan et al., 2011), 128.22-215.93 mg GAE/g using water/ethanol 70% (Ky and Teissedre, 2015) to 522.49- 546.50 mg GAE/g using a Soxhlet extractor with petroleum ether (Baydar et al., 2011). Considering this, it is extremely difficult to consider the initial TPC only based in literature results.

TPC was determined for each temperature used during HCW treatment. The results are present in figure 4.1.

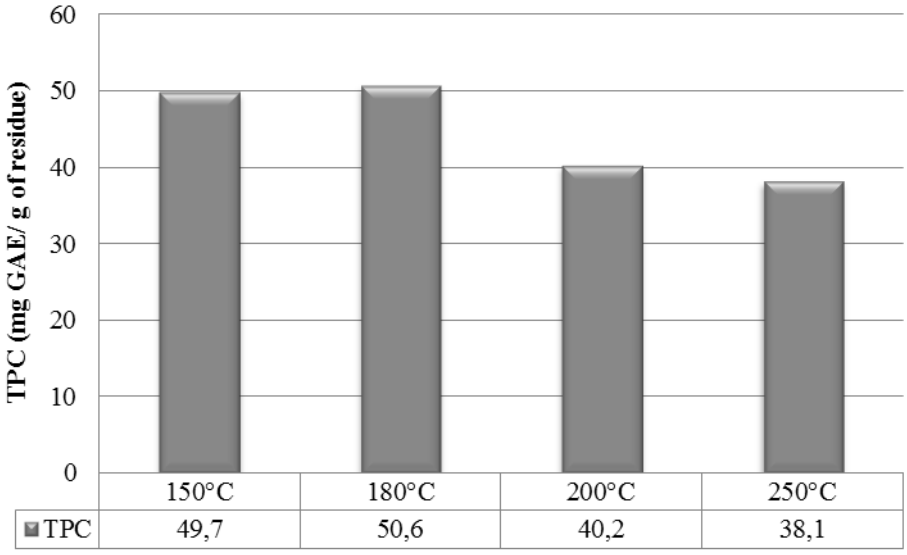


Figure 4.1: TPC obtained from Folin-Ciocalteu method performed for each temperature applied in HCW treatment at 100 bar and 5 ml/min.

Analyzing figure 4.1, it was verified a TPC in red GP range of 38.1-50.6 mg GAE/g GP being the highest content obtained for the extraction performed at 180 ºC and the lowest content recovered was obtained for the extraction performed at 250ºC. Higher temperatures promote mass transference leading to an improvement of extraction efficiency. With an increase of temperature, diffusion coefficient increase promoting sample wetting and matrix penetration (Mandal, S.C.; Mandal, V.; Das, 2015). However, as already mentioned, high temperatures could also lead to compound degradation, reducing TPC recovered since higher operational temperatures can promote the degradation of aromatic rings and the breakage of ether linkages undergoing into decarboxylation reactions in subcritical water (Machmudah et al., 2015). The

substituent groups on the ring structure, such as amino, hydroxyl and methoxyl were reported as acting as activate groups which promotes the thermal decarboxylation of benzoic acid derivatives under subcritical water conditions (Khuwijitjaru et al., 2014). Moreover, each compound degraded at a different rate as it can be seen on table 4.1.

Table 4.1: Degradation kinetics parameters for phenolic compounds in subcritical water (Khuwijitjaru et al., 2014).

Compounds	Temperature (°C)	Rate constant, $k \times 10^3$ (min^{-1})[a]
Caffeic acid	100	7.8 ± 0.6
	150	28.0 ± 2.1
Chlorogenic acid	100	6.1 ± 0.3
	150	18.4 ± 1.1
	200	42.3 ± 1.9
p-Coumaric acid	100	1.7 ± 0.3
	150	7.1 ± 0.8
Gallic acid	100	5.9 ± 0.3
	150	32.2 ± 3.8
Gentisic acid	100	4.3 ± 0.2
	150	16.1 ± 1.9
p-Hydroxybenzoic acid	100	0.7 ± 0.1
	150	3.8 ± 0.5
	200	18.8 ± 0.2
Protocatechuic acid	100	3.3 ± 0.1
	150	8.3 ± 0.3
	200	60.7 ± 2.1
Syringic acid	100	1.9 ± 0.2
	150	6.6 ± 0.9
	200	21.2 ± 0.6
Vanillic acid	100	0.8 ± 0.1
	150	1.6 ± 0.1
Catechin	100	11.7 ± 0.8
	150	20.6 ± 0.7
Vanillic acid	280–350	—

4.1.3.2. Extraction of phenolic compounds in red GP using organic solvents

Solid-liquid extraction using different solvents is the most commonly method for phenolic compounds isolation (Alothman et al., 2009). Solvent polarity plays a key role in phenolic compounds extraction since crude phenolic extracts contain complex mixtures with different classes of compounds, which are selectively soluble in the different solvents. The choice of extraction solvents such as water, acetone, ethyl acetate, alcohols (methanol, ethanol and propanol) and their mixtures will influence the yields of phenolics extracted. (Garcia-Salas et al., 2010);(Naczk and Shahidi, 2006). It is reported that higher molecular weight flavanols are better extracted with aqueous acetone (Labarbe et al., 1999) while ethanol or weak organic acids such

us citric acid are used to obtain anthocyanin-rich phenolic extracts from plant materials (Shi et al., 2005). Comparing to methanol, ethanol is less toxic and often used mostly due to health and safety reasons since it represents, at certain amounts, an edible solvent and can be obtained from renewable sources. Moreover, since ethanol is slightly less polar than methanol, a larger proportion of non-polar compounds could be extracted from the raw material when using it as extraction solvent.

Some studies showed that acetone-water system extracted considerably higher amounts of phenolic compounds than the ethanol-water or methanol-water systems for several different raw materials (Chavan, U. D. and Amarowicz, 2013; Karimi et al., 2014; Tuncel and Yilmaz, 2015)

It is very important to notice that the concentration and composition of phenolics in red wine grapes and their contents vary drastically with species, variety, maturity of the grapes and a wide range of environmental and management factors such as climate, soil conditions, cultivation region and storage conditions, etc. (Ramirez-Lopez and DeWitt, 2014). Therefore, it is expectable to obtain a wide range of phenolic composition. In table 4.2 is presented different reported content ranges for the phenolic compounds analyzed in this study.

Table 4.2: Phenolic compounds contents of several grape pomaces from different red grapes varieties.

	Content (mg/g pomace)	**Solvents**	**Literature**
Gallic acid	0.12-0.24	Acetone:water:acetic acid	Baydar *et al.* (2011)
Chlorogenic acid	0.04-0.23	MeOH:water:acetic acid	Rockenbach et al., (2011)
Caffeic acid	0.03-0.44	Acetone, MeOH, Petroleum ether.	Ramirez-lopez *et al.* (2013)
p-Coumaric acid	0.02-0.21	Acetone, MeOH, Petroleum ether.	Ramirez-lopez *et al.* (2013)
Ferulic acid	0.003-0.009	Acetone, MeOH, Petroleum ether.	Ramirez-lopez *et al.* (2014)
Resveratrol	0.007-0.255	EtOH:water:hydrochloric acid	Iacopini *et al.* (2008)

In this study, 3 different extraction solvents (ethanol, acetone and citric acid) were applied using different extraction methodologies in order to evaluate phenolics compounds extraction efficiency. In table 4.3, it can be observed the results obtained for each phenolic compound in study for the organic solvents applied during extraction.

Table 4.3: Phenolic compounds contents obtained from GP pomace extracted using several organic solvents as well as the comparison with the highest content verified when using HCW.

	(mg/g extract)			
	***Water:EtOH* (75:25 v/v)**	***Water:Acetone* (20:80 v/v)**	***Citric acid aqueous solution* (3g/L)**	***Highest content obtained using HCW***
Gallic acid	0.16	0.23	0.13	2.96
Chlorogenic acid	0.001	0.01	0.002	0.12
Caffeic acid	0.0003	0.03	0.001	0.08
p-coumaric acid	0.001	0.003	0.001	0.13
Ferulic acid	0.0002	0.003	0.0001	0.08
Resveratrol	0.0004	0.01	0.0001	0.01

Comparing the results presented in table 4.3, it can be observed that most of the contents obtained are lower than the minimum values described in table 4.2 for the phenolic compounds in study, except for the gallic content. This can be due to several factors related with residue storage as well as transportation conditions which could decrease the initial phenolic content. However, these results are used as the reference value for this specific residue and for direct comparison with the results achieved by using HCW. Actually, when comparing the results obtained using organic solvents and the contents verified when using HCW, the results obtained during HCW extraction are much more coherent with the values range presented. Gallic acid content is the only value that is extremely higher when comparing with the results obtained from literature which could be related with mass concentration in the pomace, grape variety, etc.

Phenolic compounds contents described in literature could only be taken into account as indicative values of the majority of published studies for specific raw materials. Considering this, each lignocellulosic material must be treated as unique residue since no method was

developed yet that would be considered as standard for total phenolics extraction. Optimum phenolics recovery conditions could be different from one sample to the other and relies on the type of plant and its active compounds.

The main differences in the phenolics contents obtained for the extractions using different organic solvents the literature ranges obtained using also organic solvents could be related with several operational parameters such us temperature, extraction time, solid: solvent ratio, sample matrix and particle size.

Higher phenolic compound solubility is promoted by increasing temperature. As already mentioned, with the increase of temperature, mass transfer rate increases since solvents viscosity and surface tension decreases at higher temperatures, which helps the solvents to reach the sample matrices, improving the extraction rate. However, many phenolic compounds are easily hydrolyzed and oxidized. Long extraction times and high temperatures promote undesirable reactions such as enzymatic oxidation decreasing the yield of phenolics in the extracts (Biesaga and Pyrzyńska, 2013); (Davidov-Pardo et al., 2011).

The solid: solvent ratio and the number of replicate extractions performed for each sample also affect the recovery of phenolics. Increasing the solvent-to-sample ratio promotes phenolic extraction from plant samples but determining the optimum ratio is recommended so that solvent input and saturation effects of solvent by the phenolics are minimized (Khoddami et al., 2013).

Sample matrix also strongly influence phenolic extraction from plant materials since in certain lignocellulosic matrixes the phenolics presented can be binded to other elements such as carbohydrates and proteins. Other treatments can facilitate the hydrolysis of the bounded phenolics from the matrix such as the addition of enzymes (Pinelo et al., 2008) as well as acidic and alkaline hydrolysis are also employed for the isolation of phenolics from plants (Haghi and Hatami, 2010); (Vichapong et al., 2010).

Extraction using HCW presented much higher extraction yields than when using organic solvents. This is a very good result since it proved the potential of using HCW to extract added value compounds reducing the need of using organic solvents which represents a substantial health hazard to the environment and for human health and it represents high costs for industrial processes, mainly in downstream stage. Due to the special proprieties of HCW, mainly related with chemical proprieties at a specific extraction temperature, it is possible to obtain a significant range of HCW polarities during extraction process that could have a very important role in extraction efficiency. Detailed information will be present for the extraction of phenolic compounds using HCW in the next section.

4.1.3.3. Extraction of phenolic compounds in red GP using HCW

In figures 4.2-4.7 is possible to observe extraction profiles during HCW reaction time for all the phenolics compounds in study.

Gallic acid presented the higher content from all the phenolic compounds analyzed reaching a maximum of 1085 µg/g residue at 180 ºC and 15 min of extraction time. On the other hand, resveratrol is the phenolic compound with the lower extraction yield in grape pomace, achieving a total of 5.5 µg/g residue when the extraction is performed at 200 ºC and 25 min.

At room temperature, water is highly polar being a poor solvent for most non-polar organic analytes. As the temperature increases, subcritical water proprieties change with a decrease in dielectric constant, viscosity and surface tension and an increase in the diffusion rate. Therefore, polar compounds with a high solubility in water at room conditions are extracted more efficiently at lower temperatures, whereas moderately polar and non-polar compounds require a less polar medium induced by elevated temperature (Asl, A.H.; Khajenoori, 2013). At 200 °C, water has similar properties to an organic solvent, such as methanol (Smith, 2006). For the phenolic compounds extracted from GP their polarity is as follows from high to low: gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid and resveratrol (Schieber et al., 2001).

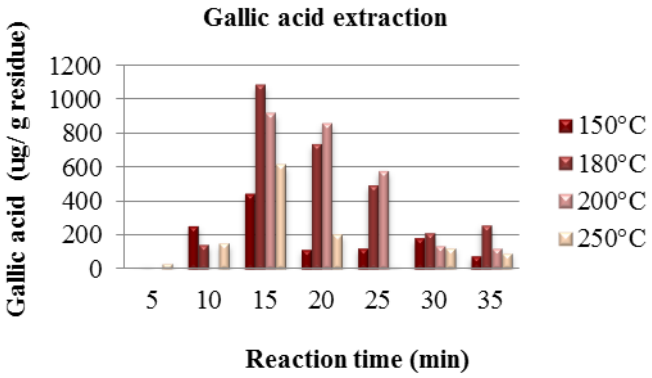


Figure 4.2: Gallic acid extraction profile along HCW treatment time.

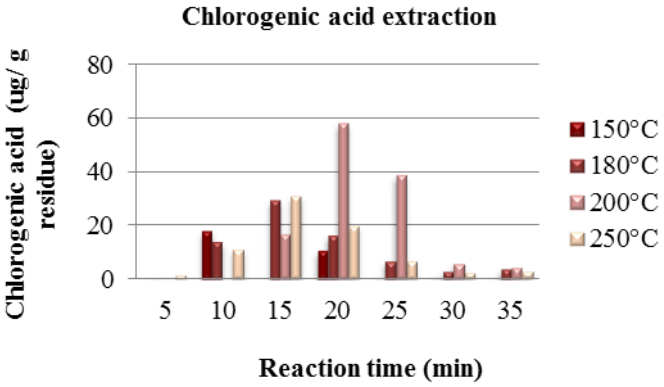


Figure 4.3: Chlorogenic acid extraction profile along HCW treatment time.

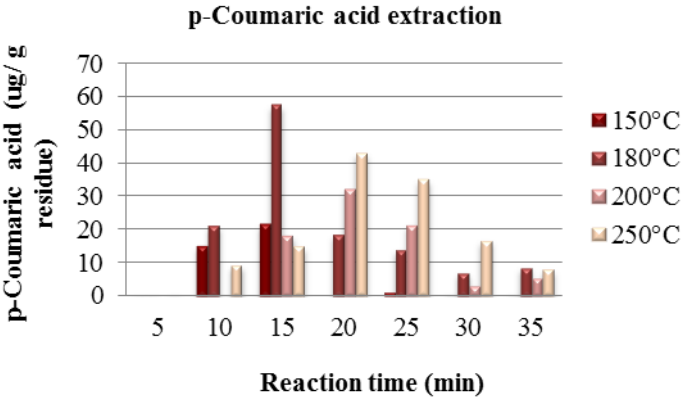


Figure 4.4: p-Coumaric acid extraction profile along HCW treatment time.

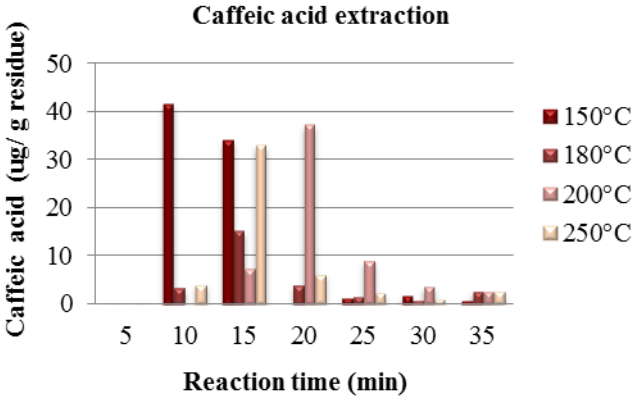


Figure 4.5: Caffeic acid extraction profile along HCW treatment time.

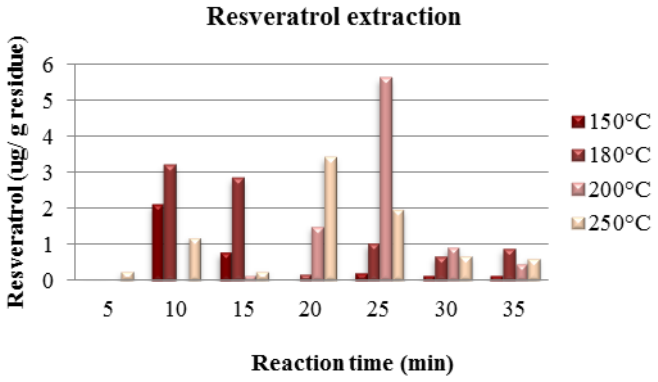


Figure 4.6: Resveratrol extraction profile along HCW treatment time.

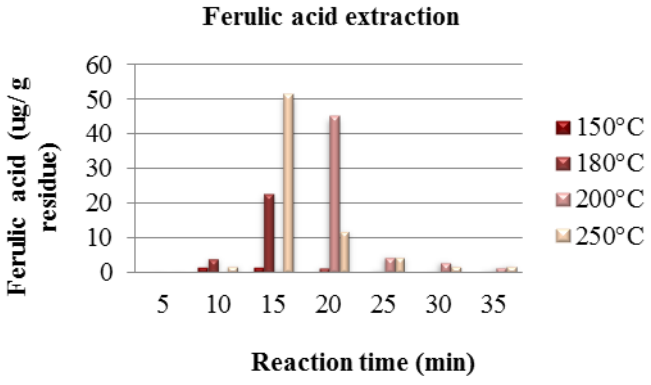


Figure 4.7: Ferulic acid extraction profile along HCW treatment time.

According with the polarity of the phenolic compounds, gallic acid would have the highest extraction yield at lower temperatures and resveratrol would have higher extraction yields at higher temperatures, as it was observed in figures 4.2-4.7. No significant differences were obtained for the remaining phenolics, which can also be related with a lower availability in the raw material. Moreover, extraction times higher than 25 min are not efficient since the low amounts recovered after that extraction time does not justify operational costs.

In table 4.4, it is possible to observe the amounts recovered for each phenolic compound during HCW extraction at several different temperatures.

Table 4.15: Phenolics compounds recovery during HCW treatment for 35 minutes.

	mg/g extract			
	150	***180***	***200***	***250***
Gallic acid	1.12	2.96	2.62	1.26
Chlorogenic acid	0.03	0.07	0.12	0.08
Caffeic acid	0.08	0.03	0.06	0.05
p-coumaric acid	0.04	0.13	0.08	0.13
Ferulic acid	0.004	0.03	0.06	0.08
Resveratrol	0.004	0.01	0.01	0.01

It is notable that gallic acid presented the highest yield of extraction for all the temperatures in study, representing more than 80% of all the extracts obtained. This is in accordance with literature since it is reported that gallic acid is one of the major phenolic compounds presented in red GP together with epicatechins and catechins, not analyzed in this study (Ramirez-Lopez and DeWitt, 2014). The lowest content was observed for resveratrol that does not exceeded 1% of the extract in all the temperatures in study.

Comparing the results obtained for phenolics compounds extraction using HCW with the contents reported in literature, it can be observed significant differences between values obtained for some of the phenolic compounds in study (table 4.2 and 4.4). The largest differences were verified for gallic and ferulic acids content that were much higher in our study when compared with the range presented in literature, nearly 10 times higher. For phenolic compounds extraction of red grape pomace and comparing with organic solvent extraction methods, HCW has about 10 times higher extraction yields (table 4.3). Analyzing all the results, phenolics compounds HCW extraction in red GP should be performed between 180ºC and

200ºC during 25 min in order to maximize extraction yield and avoiding phenolic compounds degradation.

4.1.4 Conclusions

GP phenolic compounds extraction was studied using HCW as single solvent at different operational temperatures. In order to evaluate HCW potential, extractions were also performed using conventional solvents. A TPC range of 38.1-50.6 mg GAE/g GP was determine in GP using HCW at temperature range of 150-250ºC being the highest content obtained for the extraction performed at 180 ºC and the lowest content recovered for the extraction performed at 250ºC, as expected. High temperatures can lead to compound degradation, reducing TPC recovered.

Comparing phenolic compounds extraction of red GP by using HCW or conventional extraction solvents such as methanol, ethanol or citric acid, HCW extraction performed at temperatures between 180-200ºC during 25 min has about 10 times higher extraction yields than when using conventional extraction methodologies. These results demonstrate the potential of using HCW as an alternative environment friendly extraction solvent. However, it is very important to compare the use of organic solvents and alternative solvents by using exactly the same raw material since only those can be directly compared due to the high variability of the residue.

4.2

Grape pomace as alternative carbon source for carotenoid-producing oleaginous yeast after HCW treatment

Part of the work presented on this chapter was reported in a master thesis.

4.2.1 Introduction

A significant part of world biomass energy comes from waste generated by the wood, pulp and paper industries (U.S. Energy Information Administration, 2014). But increased awareness of the need to take full advantage of material resources is driving policies towards a zero-waste economy and drawing attention to the valorization of many other waste feedstocks. The food and drink industry is the largest manufacturing sector in the EU in terms of turnover, value added and employment (FoodDrinkEurope, 2014), which shows the great potential of the food supply chain waste as a renewable biorefinary feedstock (Pfaltzgraff et al., 2013). Grape is one of the most abundant crops cultivated in Europe, Asia and America, available global grape production having reached 70 million tons in 2014, of which over 50% were pressed and fermented for making wine (OIV, 2015). Grape pomace (GP) is the highly heterogeneous residue that remains after pressing the grapes for making wine. GP represents ca. 10% by weight of the total grape input, and if the grapes are stripped from the stalks before processing, GP consists of about 40% seeds and 60% skin and pulp. Traditional uses of GP include using this residue for performing an additional fermentation or distillation to produce wine or a spirit, using the seeds and skin as a source of oil and tartaric acid, respectively, and using GP as cattle feed, or as compost. However, GP is a waste feedstock rich in lignocellulosic material, in addition to being a source of added value compounds, and the valorization of this abundant biomass waste is attracting a lot of interest (Mendes et al., 2013; Tseng and Zhao, 2013; Vergara-Salinas et al., 2015; Yedro et al., 2014). Hot compressed water (HCW) is a common designation for water at temperatures above 150 ºC and pressures above its vapor pressure (Möller et al., 2011). Relative to water at ambient conditions, the dielectric constant of HCW is lower, which increases the ability of water to solubilize hydrophobic organic compounds, such as free fatty acids or phenolics, making HCW a good extraction medium. On the other hand, the ionic product of HCW is higher, and the increased concentrations of hydrogen and hydroxyl ions make biomass hydrolysis faster, i.e. water acts simultaneously as reactant and catalyst (Brunner, 2014; Haghighi Mood et al., 2013; Ruiz et al., 2013; Wu et al., 2008).

In the present work we report on the HCW treatment of GP from the production of red wine, and the use of the resulting carbohydrates as alternative industrial carbon source for yeast growth. Three yeast strains were selected based on their relevance for industrial applications: *Saccharomyces cerevisiae*, *Rhodotorula yarrowii* and *Rhodotorula babjevae*. *Saccharomyces cerevisiae* is widely used for the production of bioethanol, and is used here as reference. *Rhodotorula yarrowii* and *Rhodotorula babjevae* are oleaginous yeasts, which can accumulate up to 65% of total dry cell weight as lipids, when grown under limited nitrogen (I. Sitepu et al.,

2014). Microbial oil represents a viable alternative to plant oil and fat (Gong et al., 2014). Microbial oil is rich in omega-3 and omega-6 fatty acids that have numerous applications in the food and pharmaceutical industries. Additionally, *Rhodotorula* genera is a natural source of pigments, mainly carotenoids (Buzzini, P.; Innocenti, M.; Turchetti, B.; Libkind, D.; van Broock, M.; Mulinacci, 2007) which are used commercially as food colorants, animal feed supplements, and in nutraceutical applications.

4.2.2 Materials and Methods

The materials and methods applied to perform this study and described in detail in the previous chapter Material and Methods. Nevertheless, a review is described below.

a. Materials

Grape pomace (GP) from the fermentation of red wine was kindly provided by a Portuguese wine producer. Grapes were destemmed before pressing. The original raw material, with ca. 60 wt.% water, was freeze-dried for 48 h, allowed to warm to room temperature and milled to ca. 2 mm particle sizes, and the resulting GP powder was stored in plastic bags kept at -18 ºC.

b. Characterization of GP powder

The average water, ash, nitrogen and lipids contents were determined according with the methods described by the National Renewable Energy Laboratory (NREL, 2014) and described in detail in the previous Materials and Methods chapter.

Defatted GP powder was used to perform a hydroalcoholic extraction to analyze free available sugars which were then quantified by high-performance liquid chromatography (HPLC). Defatted, soluble sugar-free GP powder was then submitted to a two-step acid hydrolysis (NREL, 2014), first with 72% (w/w) sulphuric acid (3 mL) for 1 h, at 30 ºC, in a water bath, and after addition of 84 mL of ultrapure water to achieve a 4% (w/w) solution of sulphuric acid, hydrolysis proceeded for 1 h, at 121 ºC, in a silicone bath. After filtration, glucose, galactose, fructose, arabinose, mannose, and xylose were quantified by HPLC, as indicated above. From the remaining residue after acid hydrolysis, its dry weight, nitrogen and acid insoluble contents were determined.

c. Hot compressed water (HCW) hydrolysis

All experiments were performed using a semi-continuous-flow apparatus.

To perform an experiment, ca. 3 g of GP powder were loaded into the reactor, which was then filled with water at ambient temperature and pressurized to 100 bar for testing for leaks and to avoid degradation of the sample upon subsequent heating. The two heating jackets were then turned on, at a heating rate of 20 ºC/min, and water started being pumped, at a flow rate of 2 mL/min. When the working temperature reached 50 ºC (after about 5 min), time count and sample collection started. The whole of the outlet stream of the reactor was collected for analysis, in a set of vials (14 mL samples), through the ca. 1 h duration of the experiment. The first samples were thus taken while temperature was approaching the desired working temperature (e.g. it took an additional 15 min for temperature to rise from 50 ºC to a working temperature of 180 ºC). When the liquors cooled down to ambient temperature, some precipitation occurred. The liquors were filtered and stored at 4 ºC while waiting for analysis.

All experiments were performed at a constant pressure of 100 bar.

The GP residue that remained in the reactor after hydrolysis with HCW was washed and dried in an oven at 105 ºC for 4 h, and weighed.

d. Characterization of the liquors and the residue obtained after hydrolysis with HCW

The amount of water soluble compounds in the liquors was determined by freeze-drying of samples collected throughout the HCW experiments and weighing the resulting powder.

The total amount of carbohydrates in the hydrolysates was determined using the phenol-sulphuric acid colorimetric method (Dubois et al., 1956). The monosaccharides in the hydrolysates were quantified by HPLC, as indicated earlier. The amount of oligosaccharides in the hydrolysates was estimated by subtracting the amount of monosaccharides in the hydrolysates from the total amount of carbohydrates. A correction was made based on the amounts of degradation compounds, determined as indicated below. The hydrolysates obtained at 180 ºC were submitted to a more detailed analysis of carbohydrates. To this end, an aqueous solution of the hydrolysates was submitted to enzymatic digestion with Viscozyme L at pH 5.5, at 55 ºC, for 3 days. Samples were taken, filtered, and the monosaccharides were quantified by HPLC.

The quantification of degradation compounds – furfural and 5-HMF – directly in the liquors was carried out by HPLC with UV detection at 280 nm (Schieber et al., 2001).

The quantification of acetic acid was also carried out directly in the liquors, using a Dionex ICS-3000 system with a PDA ICS Series - 210 nm detector, using a 7.8 x 300 mm BIORAD

Aminex HPX-87H column with pre-column, at a constant temperature of 30 ºC. A 10 mN H_2SO_4 solution was used as mobile phase, at a constant flow rate of 0.6 mL/min.

The GP residue that remained in the reactor after hydrolysis with HCW was submitted to a two-step acid hydrolysis with sulphuric acid, as done to characterize GP powder.

e. Yeast growth

The *Saccharomyces cerevisiae*, *Rhodotorula yarrowii* and *Rhodotorula babjevae* yeast strains were from the Portuguese Yeast Culture Collection (PYCC-UCIBIO). The *R. yarrowii* (PYCC 5629) and *R. babjevae* (PYCC 4781) strains were isolated from strawberry tree fruit and leaves substrates, respectively. *Saccharomyces cerevisiae* (PYCC 4889) was isolated from wine lees from a distillery.

Each strain was assessed for its ability to assimilate a mixture of hexoses (glucose, fructose, galactose) and pentoses (arabinose, xylose) identified in the hydrolysates, combined in different proportions. The screening was performed in 96-well microplates. The culture medium contained (per liter) 6.7 g of Yeast Nitrogen Base minimal medium and 5 g of the carbohydrates under study. The culture medium components were sterilized by filtration. All the growth experiments were performed at 25 ºC for 2 days, with shaking at 120 rpm. Yeast growth was monitored by optical density (OD) measured at 630 nm over time. The data reported are the result of replicate experiments.

These studies were followed by others in which the standard carbohydrate mixture was replaced with GP hydrolysate, in such an amount as to yield a concentration of 5 g/L in sugar monomers.

4.2.3 Results and discussion

4.2.3.1. HCW treatment of GP

GP is a highly heterogeneous residue made up of grape bodies with seeds, and stalks. Its composition depends on the differences in the chemical composition of its constituents, as well as the proportions in which they are combined. The average composition of the GP powder used in this work is given in Table 4.5.

Table 4.5: Composition of GP powder.

Component	Relative amount ($g/100\ g_{GP}$)
Carbohydrates, soluble	2.5 ± 0.3
glucose	1.2
fructose	1.3
Carbohydrates, structural	26.6 ± 2.0
glucose	15.4
xylose	4.2
arabinose	2.6
mannose	2.2
galactose	2.2
Protein	14.7 ± 1.5
Lipids	11.9 ± 1.4
Acid insoluble lignin	31.9 ± 3.2
Ash	6.2 ± 0.5

In general, the composition of GP as regards the main constituents included in the table is within the range of values found in the literature (Deng et al., 2011; Llobera and Canellas, 2007; Mendes et al., 2013). GP from red grapes is reported to be richer in crude protein, lipids, ash and acid insoluble lignin than GP from white grapes. On the other hand, the amount of soluble carbohydrates is markedly lower for GP from red grapes than for GP from white grapes, due to the fact that when making red wine, grapes are fermented together with the juice. The carbohydrates present in GP are essentially made up of six sugar monomers, as indicated. HCW reactions were performed at four different temperatures: 150 ºC, 180 ºC, 200 ºC and 250 ºC. As shown in Figure 4.8, increasing temperature led to decreasing values of GP residue recovered (dark gray bars in the figure), and thus to increasing values of GP conversion, since increasing temperature causes an increase in the severity of hydrolysis conditions, namely through an increase in the ionic product of water (Brunner, 2014).

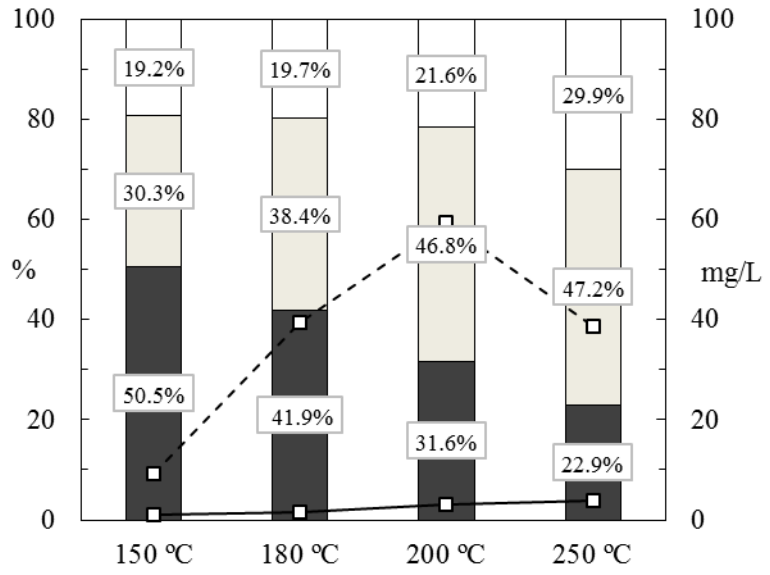


Figure 4.8: Hydrolysis of GP at different temperatures and 100 bar. Left Y axis (with data labels): Amounts of GP residue remaining in reactor after hydrolysis (dark gray bars), of water soluble compounds recovered as lyophilized hydrolysate (light gray bars), and of unrecovered material (white bars), given as wt.% of GP powder treated in the reactor. Right Y axis (no data labels): Concentration of furfural (full line) and 5-HMF (broken line) determined in the liquors.

This was accompanied by an increase in the amount of water soluble compounds recovered as hydrolysate (light gray bars), although not proportionately. Lack of proportionality is due to the fact that the amount of material not recovered as residue or water soluble compounds (white bars) increases with temperature. E.g. at 250 ºC, the loss of material reached almost 30% as might be explained by the formation of volatile and degradation compounds. In order to elucidate this difference, two of the degradation compounds commonly found in lignocellulosic

hydrolysates, namely 5-HMF, resulting from the degradation of hexoses, and furfural, produced upon degradation of pentoses (Wettstein et al., 2012)(Wu et al., 2008), were monitored in the hydrolysate. Both can hinder yeast growth (Kim et al., 2014; I. Sitepu et al., 2014; Sitepu et al., 2013; Zhao et al., 2012). As Figure 4.8 shows, the amount of furfural detected was always low, although it increased with temperature, whereas the amount of 5-HMF increased markedly with temperature up to 200 ºC, and then decreased. The hydrolysis of biomass also leads to the formation of carboxylic acids, which can likewise inhibit yeast growth (I. Sitepu et al., 2014), in addition to having an acidifying effect (Brunner, 2014; Möller et al., 2011). This may promote the occurrence of side reactions with xylose, furfural and other reaction intermediates, to form soluble polymers and insoluble humins, thereby explaining the low yields of furfural (Wettstein et al., 2012). 5- HMF has been shown to be the major degradation product from the decomposition of glucose (Möller et al., 2011). These authors observed an increase in the yields of 5-HMF with the increase in glucose conversion, but above a certain temperature the yields decreased due to the decomposition of 5-HMF to products such as levulinic acid and soluble polymers, resulting in lower overall yields in 5-HMF at 250 ºC than at 200 ºC (Möller et al., 2011). Consistent with the occurrence of thermal degradation of hydrolysis products, Figure 4.9 shows that the fraction of carbohydrates recovered in the hydrolysates relative to the amount initially present in GP powder (broken line in the figure) first increases with temperature and then decreases. This leads to a marked decrease in the amount of carbohydrates found in the hydrolysates (full line), which is already lower at 200 ºC than at 150 or 180 ºC.

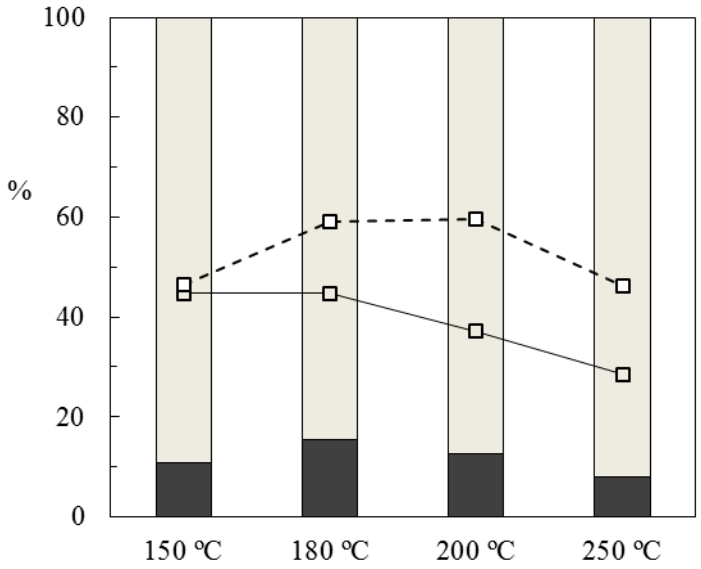


Figure 4.9: Fraction of carbohydrates in the hydrolysate as a function of the temperature of hydrolysis, at 100 bar: Monosaccharides (dark gray bars) and oligosaccharides (light gray bars). The full line represents the amount of carbohydrates in the hydrolysates (g/100 $g_{hydrolysate}$), and the broken line indicates how much of the total amount of carbohydrates of GP powder are recovered in the hydrolysates.

As shown also in Figure 4.9, most of the carbohydrates recovered in the hydrolysates are in the form of oligosaccharides. The highest amount of monosaccharides was obtained in the experiments performed at 180 ºC, the temperature at which the balance between hydrolysis of biomass and formation of sugar-derived degradation products is more favorable from the standpoint of the purpose of this work.

Figure 4.10 shows the proportion of the six sugar monomers identified in the GP hydrolysates obtained at 180 ºC, after enzymatic hydrolysis to convert oligo- into monosaccharides.

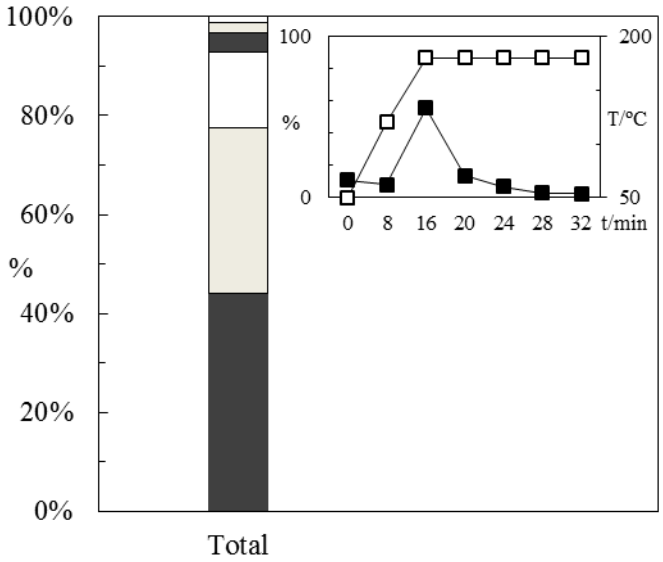


Figure 4.10: Composition profile of the hydrolysates obtained at 180 ºC and 100 bar, in sugar monomer equivalents after enzymatic digestion. From bottom to top (highest to lowest wt.%), these are: fructose, glucose, arabinose, galactose, mannose, and xylose. The inset shows how the total amount of monosaccharides recovered in the liquors is distributed throughout the duration of the assay (filled symbols, left Y-axis), as well as the evolution of the temperature inside the reactor (empty symbols, right Y-axis). The lines are guiding lines.

Only a very small amount of fructose exists in GP powder. The high levels of this compound that are found in the hydrolysates indicate that it is formed during hydrolysis. As noted by some authors (Möller et al., 2011), the high temperature and increased ionic product of HCW lead to the conversion of glucose to fructose. Considering the composition of GP powder given in Table 4.5, the results obtained at 180 ºC indicate that a considerable degree of depolymerization of GP occurred, ca. two thirds of the glucose, as such or in the form of fructose, and nearly all of the arabinose of GP powder being recovered in the hydrolysate, in agreement with a reportedly significantly slower degradation of the latter monomer in comparison with xylose/galactose (Brunner, 2014). The rest of the glucose is found in the GP residue that remains in the HCW reactor.

The inset of the figure shows that most of the monosaccharides found in the hydrolysates are obtained from liquors exiting the reactor once the temperature reaches the target temperature of the assay. A similar trend was observed for the other temperatures tested. Due to experimental constraints, it takes a certain length of time for temperature to reach the working temperature of interest. But once it is reached, biomass hydrolysis is fast, and hence the amount of carbohydrates released reaches a peak within the first 20 min, without the need to prolong the assay for much longer.

4.2.3.2 GP hydrolysate as carbon source for yeast growth

The main goal of this work is to assess the potential of HCW to hydrolyze GP and produce liquors to be used as carbon and energy source for yeast growth. To this end, the hydrolysis of GP needs to accomplish the conversion to high molecular weight sugar polymers, not assimilable by yeast, into simpler sugars, without the production of inhibitory amounts of toxic by-products. Simple sugars are readily or exclusively assimilated by most yeast. Glucose is the preferred carbon source, although fructose (Leandro et al., 2009), galactose and some pentoses are also readily catabolized by many potentially interesting yeasts, including several *Rhosdosporidium* and *Rhodotorula* species (Leandro et al., 2009). Higher saccharides are less often assimilated, their utilization depending on whether yeasts have the glycosidases required to perform the digestion of the oligosaccharides resulting from incomplete GP hydrolysis. Three yeast species, selected essentially according to their biotechnological potential and the substrate of isolation, were used, namely *Saccharomyces cerevisiae*, the widely employed bioethanol producing yeast, and two red oleaginous basidiomycetous species, namely *Rhodotorula yarrowii* and *Rhodotorula babjevae*, isolated from fruit and leaves, respectively. Yeast growth was first assessed using simple sugar mixtures mimicking the relative abundance obtained at the different hydrolysis temperatures (Table 4.6). All the mixtures had a glucose to fructose mass ratio of 1. Mixture S1 combined glucose, fructose and arabinose, the most abundant sugars in the hydrolysates. Mixture S4 combined all the sugar monomers detected in the hydrolysates, with the exception of mannose, also on a 1:1:1:1:1 ratio. Mixtures S2 and S3 provided intermediate combinations. The total sugar concentration was kept constant at 5 g/L. Considering the ultimate goal, two parameters were taken into account in this assessment: growth rate, and the OD630nm at 72 h as a measure of the yeast cell density achieved at each condition. *R. babjevae* is known to assimilate the five sugar monomers tested, unlike *R. yarrowii* that does not grow on arabinose, and shows delayed growth on galactose and xylose (PYCC databank). In this context, the latter species is even more restricted than *S. cerevisiae*, which does not assimilate arabinose or xylose (Leandro et al., 2009), but grows on galactose. The results obtained for assays with standard sugar mixtures generally agree with a greater

amount of assimilable sugars available to *R. babjevae* than to *S. cerevisiae*, and even more so when compared to *R. yarrowii*. To evaluate the potential of GP hydrolysates as sole carbon source for yeast growth, the GP hydrolysate was used in such an amount as to ensure that the concentration of monosaccharides in the growth medium was 5 g/L. When cultured on standard media, the yeast strains grew exponentially at a constant rate until they reached a stationary phase, after less than 24 h (Table 4.6). When grown on GP hydrolysates as sole carbon source, biomass increased steadily but comparatively much more slowly, probably due to a harsher growth medium. However, after 72 h this steady growth led to OD values that were similar, or more usually higher, than those achieved with the standards.

Table 4.6: Effect of the carbon source on the growth rate (μ) and optical density (OD) at 72 h for yeast strains.

	Saccharomyces cerevisae *PYCC 4889*		*Rhodotorula yarrowii* *PYCC 5629*		*Rhodotorula babjevae* *PYCC 4781*	
	OD (72 h)	μ/h^{-1}	OD (72 h)	μ/h^{-1}	OD (72 h)	μ/h^{-1}
Standards						
S1	0.24 ± 0.05	0.14 ± 0.05	0.19 ± 0.01	0.19 ± 0.01	0.31 ± 0.05	0.18 ± 0.01
S2	0.23 ± 0.06	0.12 ± 0.06	0.20 ± 0.03	0.13 ± 0.01	0.32 ± 0.05	0.21± 0.02
S3	0.23 ± 0.05	0.14 ± 0.03	0.21 ± 0.01	0.18 ± 0.02	0.31 ± 0.04	0.17 ± 0.03
S4	0.28 ± 0.04	0.16 ± 0.01	0.22 ± 0.04	0.10 ± 0.01	0.32 ± 0.01	0.19 ± 0.01
GP hydrolysates						
T=150 ºC	0.35 ± 0.01	0.02 ± 0.01	0.32 ± 0.04	0.05 ± 0.01	0.84 ± 0.13	0.05 ± 0.01
T=180 ºC	0.38 ± 0.01	0.02 ± 0.01	0.31 ± 0,05	0.05 ± 0.01	0.74 ± 0.17	0.04 ± 0.01
T=200 ºC	0.23 ± 0.01	0.02 ± 0.01	0.17 ± 0.02	0.02 ± 0.01	0.57 ± 0.07	0.04 ± 0.01
T=250 ºC	0.29 ± 0.02	0.02 ± 0.01	0.23 ± 0.01	0.02 ± 0.01	0.28 ± 0.06	0.03 ± 0.01

S1 to S4 are mixtures of monosaccharides: S1 = Glucose(Glu)/ Fructose(Fru)/ Arabinose(Ara) (1:1:1); S2 = Glu/ Fru/ Ara/ Galactose(Gal) (1:1:0.5:0.25); S3 = Glu/ Fru/ Ara/ Gal/ Xylose(Xyl) (1:1:0.5:0.5:0.25); S4 = Glu/ Fru/ Ara/ Gal/ Xyl (1:1:1:1:1).

In addition to monosaccharides in amounts identical to those found in the standard sugar mixtures, the GP hydrolysate medium contained oligosaccharides, as well as degradation compounds and acidic species, such as acetic acid. As seen earlier, both furfural and 5-HMF were identified in the hydrolysates, and both can have negative impact on yeast growth (Kim et

al., 2014)(I. Sitepu et al., 2014) (Zhao et al., 2012). The amount of furfural detected in the GP liquors was low, but that of 5-HMF was comparatively much higher. Even so, the concentration of these compounds in the liquors was much lower than that shown to impact negatively on the growth of many oleaginous yeast species (I. Sitepu et al., 2014). So was the concentration of acetic acid, which analysis of the liquors revealed not to exceed 14 mg/L, much lower than the value that caused severe growth inhibition of *R. babjevae* strains (I. Sitepu et al., 2014). In addition to furfural, 5-HMF and acetic acid, compounds released upon degradation of lignin (Brebu and Vasile, 2010) have also been shown to inhibit the growth of yeast species, in particular low molecular weight lignin degradation products (Zhao et al., 2012). Thermal degradation products may also have synergistic effects (Zhao et al., 2012). In assays with GP hydrolysates obtained at temperatures above 180 ºC, a higher amount of hydrolysate is needed to attain the sought 5 g/L concentration in the culture medium (Figure 4.9, full line), and thus higher inhibitor concentrations are also attained. This may explain the poorer performance exhibited by all yeast strains grown under these conditions. On the other hand, the amount of GP hydrolysate that yielded a 5 g/L concentration of monosaccharides in the culture media contained an additional carbon source in the form of more complex carbohydrates. The results of Table 4.6 (OD 72 h) must reflect the ability of each species to assimilate the sugar polymers that were small enough to be recovered as water soluble compounds in the liquors, such as dimers and trimers. *S. cerevisiae* is able to assimilate glucose dimers, such as maltose, or the glucose trimer maltotriose (Salema-oom et al., 2005), but not cellobiose or cellotriose, which are formed in the digestion of cellulose. And being a fermenting yeast, *S. cerevisiae* will shift a high amount of carbon to produce ethanol directly instead of producing biomass. In addition, and in line with our observations, it has already been shown that toxic by-products resulting from HCW hydrolysis of lignocellulosic materials considerably inhibit *S. cerevisiae* growth and ethanol production (Jayakody et al., 2012). Non-Saccharomyces yeasts are known to produce extracellular hydrolytic enzymes that can degrade polysaccharides (Gong et al., 2014; Strauss et al., 2001). For example, the yeast *Cryptococcus curvatos* was shown to grow and produce lipids from β-1,4 glucans and β-1,4-xilans derived from incomplete hydrolysis of cellulose and hemicellulose. As referred earlier, *R. yarrowii* is more limited than *R. babjevae* as regards carbon sources, and this includes its inability to assimilate cellobiose (PYCC databank). The higher versatility of *R. babjevae* in this respect must be behind its comparatively much better performance, as evidenced by an OD that is over twice as high for hydrolysates obtained at 180 ºC than for any of the standard sugar mixtures. The results obtained clearly reflect the ability of this species to grow on more complex carbohydrates, confirming the applicability of GP hydrolysates a sole carbon source.

4.2.4 Conclusions

HCW technology allows fast recovery of up to 60% of structural carbohydrates of grape pomace, with low levels of sugar degradation. Most of the carbohydrates recovered are in the form of oligosaccharides, which can be further digested by enzymes or used directly by microorganisms such as Rhodotorula babjevae yeast. This yeast exhibited an OD 2.5-fold higher than obtained with model mixtures, demonstrating, for the first time, that HCW-treated agricultural residues afford suitable carbon source for the growth of non-Saccharomyces yeast. GP hydrolysates can thus be used as a cheaper, alternative carbon source, for microbial growth and production of added-value compounds.

5

Oleaginous yeast potential to assimilate alternative carbon sources produced from apple pomace HCW treatment

Part of the results presented in this chapter was reported in a master thesis.

5.1. Introduction

Apples are the fourth most widely produced fruit in the world after bananas, oranges and grapes (Malik, 2013). Considering the worldwide apple production, 71% is consumed as fresh apple for human consumption while about 20% is processed into added-value products. Concentrate apple juice represents 65% of the total added-value products obtained from apples being the remain processed into apple juice, apple wine, cider, apple purees, jams and dried apple products (Shalini and Gupta, 2010).

Apple pomace (AP) is the remaining solid residue after juice extraction from fresh apples and consists of a heterogeneous mixture of pulp, seeds and peels. The conventional process for juice recovery removes 75% of fresh weight as juice and 25% as pomace (Mahawar et al., 2014). AP is a by-product generated not only by cider industry but also from several other industries such brandies production, distilled or spirits and vinegars industries (Sato et al., 2010). Based on 75% juice extraction efficiency, about 27 million kilograms of apple pomace is generated annually as the primary waste product (Baiano, 2014). The disposal of this high volume represents a major issue for apple processors and, often, is dried to produce stable cattle feed (Shalini and Gupta, 2010).

Similar to grape pomace (described in chapter 4), AP is a lignocellulosic residue mainly composed of hemicellulose and cellulose connected by lignin polymers. The treatment of AP with HCW led to the hydrolysis of those complex polysaccharides into simpler oligosaccharides, which could be assimilated by yeasts.

The general chemical composition of apple pomace is presented in table 5.1.

Table 5.1: Chemical composition of apple pomace (Sato et al., 2010).

APPLE POMACE	
Composition	Content
Moisture (%)	11.43
Ash (%)	1.80
Lipids (%)	1.53
Proteins (%)	2.74
Total polyphenols g.kg^{-1}	4.61
Total carbohydrate g.100g^{-1}	39.35
Dietary fiber	43.63

Several studies have shown the AP potential as a carbon source for microorganisms to produce added-value products such as citric acid (Hoseyini et al., 2011), lactic acid (Kosseva and Webb, 2013), ethanol and acetic acid (Parmar and Rupasinghe, 2013). In this chapter, AP treated by HCW is tested as low cost and sustainable carbon source for lipid and carotenoids production by yeast.

5.2. Materials and Methods

Materials and methods were described in detail in Chapter 2: Materials and Methods. A brief review is presented here.

5.2.1. Apple pomace hydrolysis using HCW

To study the temperature effect on AP hydrolysis, experiments were performed at stepwise temperature increase at 50 °C, 70 °C, 90 °C, 130 °C, 150 °C, 170 °C, 190 °C, 210 °C, 230 °C and 250 °C though a single experiment at 100 bar with a water flow rate of 5 ml/min.

In a second stage, hydrolysis experiments were performed at four different temperatures, 130 ºC, 150 ºC, 170 ºC and 190 ºC, in which liquors were collected each 20 min. All other conditions were maintained as described above.

5.2.2. Liquors analysis

All the liquors analysis including WSC's, total carbohydrates, monosaccharides, degradation compounds and total phenolic contents were performed as previously described under Chapter 2: Materials and Methods.

Total oligosaccharides content was obtained by decreasing the monosaccharides content obtained by HPLC from the total amount of carbohydrates determined by the colorimetric method. It is important to highlight that the HPLC column used did not separate xylose from galactose and fructose from arabinose due to similar retention times.

5.2.3. Yeast growth with AP hydrolysates

Five different oleaginous yeasts strains supplied by the Portuguese Yeast Culture Collection (PYCC-UCIBIO) were used in this study: *Rhodotorula babjevae*, *Rhodotorula toruloides*, *Rhodotorula glutinis, Rhodotorula mucilaginosa* and *Yarrowia lipolytica.*

The first trial to test the potential of using AP hydrolysates for yeasts growth was done with a blend of all liquors obtained through the temperatures from 50ºC to 250ºC obtained in section 5.2.1. as the sole carbon source on Yeast nitrogen medium (YNB) at 12g/L of total monosaccharides. Growth conditions were described in detail in Chapter 2: Materials and Methods.

Secondly, the same set of yeast were assayed on AP hydrolysate blend mentioned above using a specific medium for lipid production at 70 g/L of hydrolysate (Zhao et al., 2008) .

5.2.4. Yeast growth and lipid accumulation using optimized AP extract

Rhodotorula mucilaginosa was grown using AP hydrolysates obtained at 130 ºC, 150 ºC, 170 ºC and 190 ºC and the specific medium for lipid accumulation, as described above.

The scaled-up experiment using *Rhodotorula mucilaginosa* and AP hydrolysate obtained at 130 ºC was as described except that was carried out in 50 mL erlenmeyer flasks with 10 mL of medium.

5.2.5. Lipids analysis

Intracellular lipid droplets were observed by fluorescence microscope in cells grown to stationary phase, t= 55h (by nitrogen starvation) stained with Nile red.

5.2.6. Carotenoids extraction and analysis

Carotenoids were extracted and analyzed in terms of total carotenoids by spectrometry and β-carotene content by HPLC from cells grown to stationary phase (by nitrogen starvation).

5.3. Results and discussion

AP was hydrolyzed using HCW to obtain liquors, which were used as carbon source for oleaginous yeasts growth. Considering that an extensive description of HCW treatment was already provided in chapters 3 and 4, the application of this treatment to apple pomace will be summarized. The HCW treatment and the chemical analysis followed the same methodologies but the main goal was to set the optimum temperature at which the liquors obtained were optimum for the growth of yeast and the production of lipids and carotenoids.

5.3.1. The temperature effect on AP HCW hydrolysis

Firstly, a continuous assay was performed to evaluate the temperature influence on the hydrolysis parameters. Temperature was increased from 50 to 250 ºC, by steps of 20 ºC. At each step temperature was mantained. Liquor samples were collected after the 20 min period. Altogether, 11 samples were collected at different temperatures (Figure 5.1).

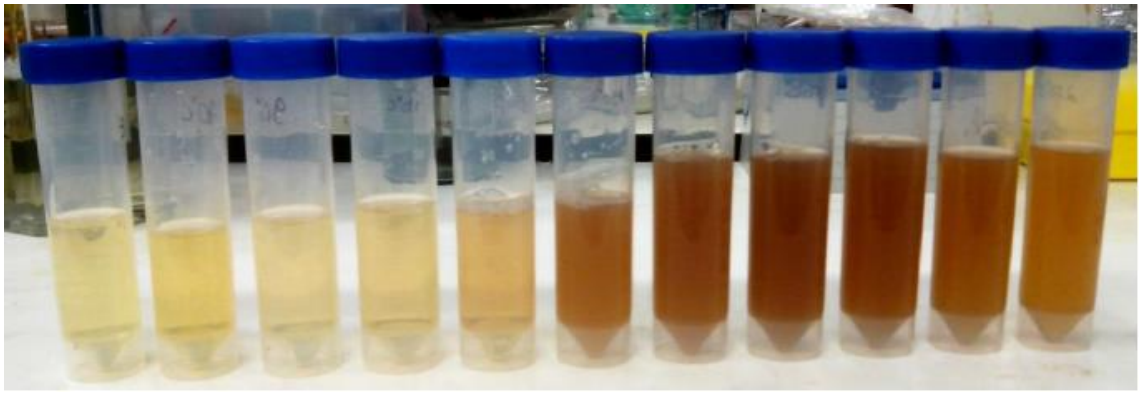


Figure 5.1: Liquors recovery during hydrolysis assay. Temperature increase from left (50 ºC) to right (250 ºC).

It is important to notice that, although the HCW's flow rate was kept constant throughout the experiment, the collected volume increased, especially for those samples collected at the higher temperatures (Figure 5.1). At higher temperatures, the heating rate is lower, which results in higher sample volumes. It is also interesting to note the differences in sample color between liquors. At temperatures higher than 150 ºC, sample start gaining a brownish color, suggesting the occurrence *of Maillard* or caramelization reactions. The *Maillard* reaction or *Maillard* browning is an acid-based catalyzed complex network of reactions between an amino acid (free or combined in proteins or peptides) and a reducing sugar, which usually occur at temperatures above 140 ºC, yielding a final polymeric and colored molecule called melanoidins (Echavarría et al., 2012), (Melville L. Wolfrom, Naoki Kashimura, 1974). At even higher temperatures, caramelization and subsequently pyrolysis begin to occur. Caramelization reaction results from the carbohydrate oxidation with the release of volatile chemicals with a characteristic caramel odor (Dziedzic, S.Z.; Kearley, 1995). Considering this, it is likely that during the HCW hydrolysis a fraction of the oligosaccharides, initially present in the lignocellulosic structure, undergoes these parallel reactions. However, some researchers have shown that microorganisms, mainly bacteria and yeasts, are capable of assimilating meladoinins and, for instance decolorized distillery wastes (Kuhad, R.C.; Singh, 2013). Though relevant, these parallel reactions were not quantified in this study.

Liquors were characterized for WSC's, total carbohydrates, monosaccharides profile, total polyphenolic and degradation compounds, namely 5HMF and furfural.

WSC's were determined after freeze-drying. As shown in figure 5.2, the soluble compounds increase with temperature, achieving a maximum value at 130 ºC from which starts to decrease reaching a minimum at 250 ºC. The breakage of glycoside bonds is favored by increasing temperature yielding soluble oligosaccharides. Considering this, the results are indicative of hydrolysis extension since higher hydrolysis extension, higher the amount of dissolved material. However, higher temperatures also promote degradation reactions as well as the formation of lignin secondary compounds derived from lignin repolymeriẓation as described in the previous chapters.

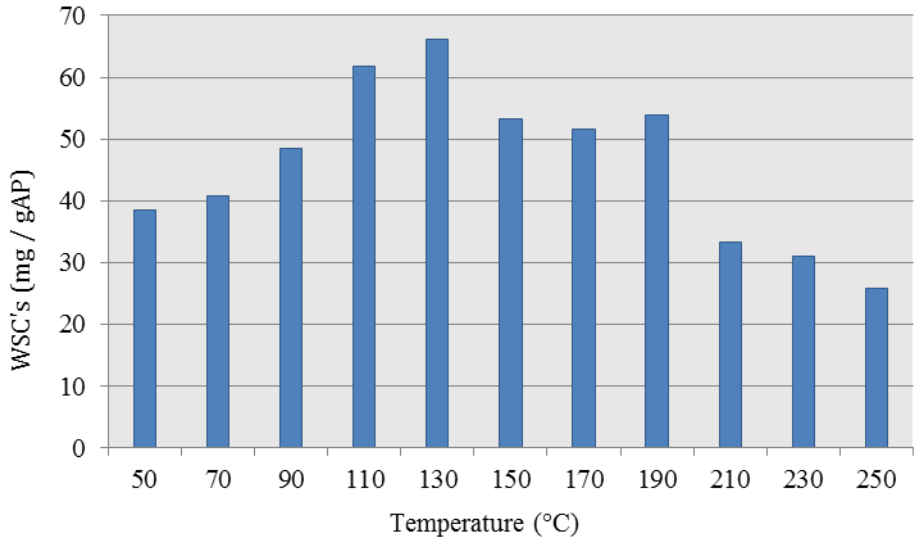


Figure 5.2: Effect of hydrolysis's temperature on the recovery of water soluble compounds from AP.

Thereby, the highest amount of WSC's was obtained within the temperature range of 110 – 190 ºC reaching more than 50% of total WSC recovered.

It is important to notice that the method used to determine the total amount of carbohydrates recovered in the liquors quantifies the total amount of carbohydrates (mono-, di-, trisaccharides, etc.) and also their degradation compounds (5-HMF and furfural) that are solubilized. The actual amount of carbohydrates was determined by subtracting the amount of degradation compounds previously determined by HPLC analysis. Monosaccharides profile was obtained by HPLC analysis. The difference between the amount of total carbohydrates (obtained from the colorimetric method) and monosaccharides (obtained from HPLC analysis) represents the fraction of oligosaccharides structures resulted from HCW hydrolysis (trisaccharides, disaccharides, etc.) which hereafter are referred as total oligosaccharides.

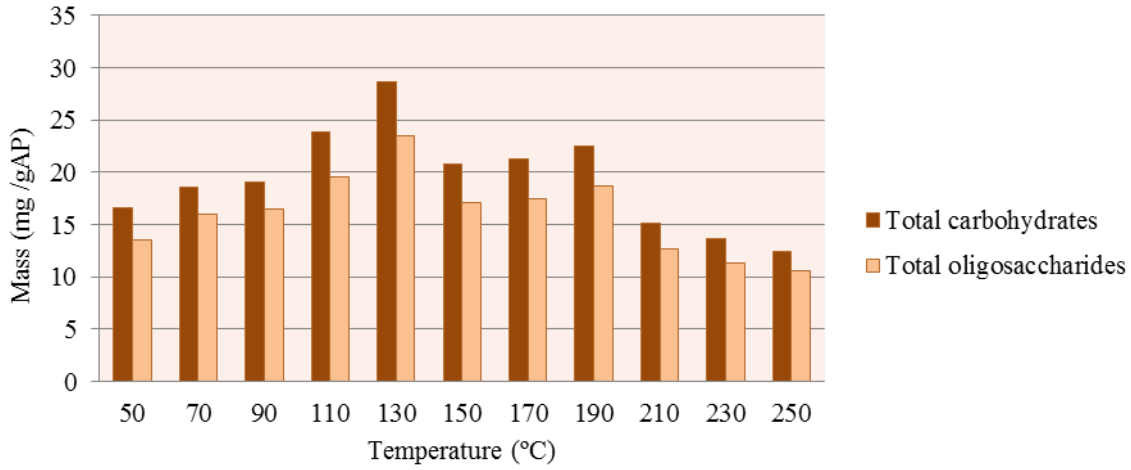


Figure 5.3: Effect of hydrolysis's temperature on the recovery of total carbohydrates and oligosaccharides recovery from AP.

In line with what was observed for WSC's, the highest conversion of carbohydrates occurs within the 110 to 190 ºC range (Figure 5.3). And, among them, oligosaccharides were the highest fraction being the remaining difference between total carbohydrates and oligosaccharides bars height representative of the amount of monosaccharides obtained at each temperature (figure 5.4).

Fructose is the major monosaccharide available in apple pomace followed by glucose and minor amounts of xylose, galactose and arabinose (Gullón et al., 2007). Accordingly, fructose and glucose are the major monosaccharides present in liquors at lower temperatures (Figure 5.4).

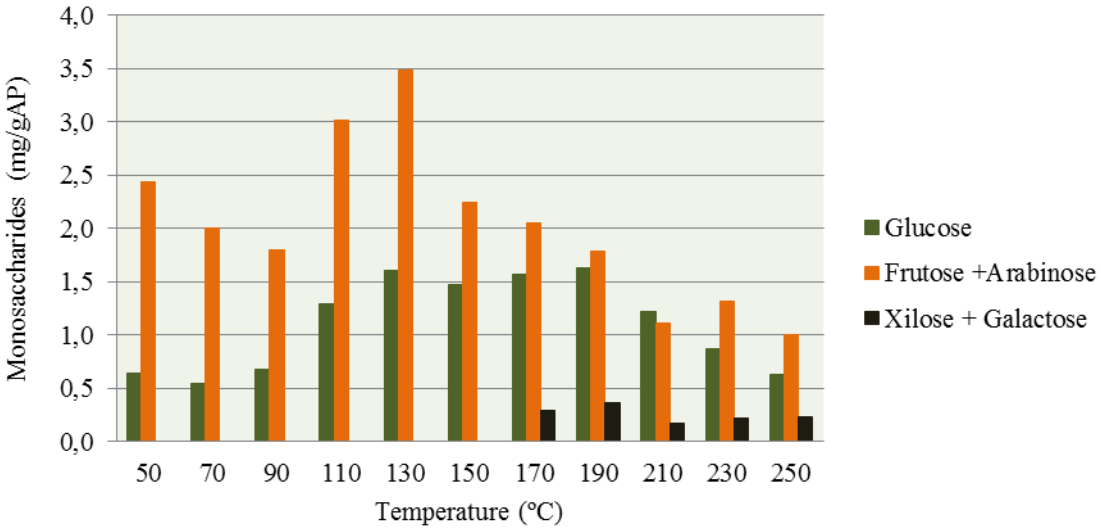


Figure 5.4: Effect of hydrolysis's temperature on the recovery of monosaccharides from AP. Xylose and galactose as fructose and arabinose co-eluted and were quantified as xylose and fructose respectively.

Fructose units are not present in hemicellulose structure, which are composed of around 500–3000 monomer units and acidic groups derived from xyloglucans, xylans, glucomannans and galactoglucomannans (Ebringerová, A.; Hromádková, Z.; Heinze, 2005). However, fructose may result from glucose isomerization, as already mentioned in Chapter 1. Due to a higher ion product and lower dielectric constant, HCW is considered an acid–base catalyst promoting hydrolysis and isomerization reactions (Gao et al., 2014; Patrick et al., 2001; Simsek Kus, 2012). Several studies reported that glucose, mannose and fructose can be mutually isomerized through an alkali-catalysis-like pathway in HCW (Lü and Saka, 2012; Usuki et al., 2007). The isomerization of glucose to fructose in sub- and supercritical water is more prevalent than its reverse reaction (B M Kabyemela et al., 1997a; Srokol et al., 2004; Watanabe et al., 2005). These facts can explain the increment observed in fructose content at the temperature range of 110 - 170 ºC.

Above 170 ºC, fructose content decreased but that can be related to its further degradation to 5-HMF. 5-HMF formation from dehydration reaction is more effective from fructose than from glucose (Despax et al., 2013). Xylose was observed only in the hydrolysates recovered at temperatures higher than 170 ºC (Figure 5.4). According to the literature, hemicellulose solubilization and hydrolysis in HCW occurs at temperatures close to 180 ºC (F. Jin, 2014) . Likewise, the results show that no xylose was detected until 170 ºC. The highest amount of xylose was recovered at temperatures between 170 ºC and 190 ºC, after which, xylose is probably degraded into furfural (Ebringerová and Heinze, 2000).

Hydrolysis of lignocellulosic material using HCW is a very complex mechanism due to the fact that several secondary reactions can occur generating a large variety of different compounds, mainly intermediate complex compounds and degradation by-products. Although research interest is increasing in the last years, until now, information is still scarce regarding the impact, stress and tolerance of certain intermediate compounds and lignocellulose degradation products in the growth and lipid accumulation performance of oleaginous microorganisms. Several degradation compounds are formed during lignocellulosic treatment including carbohydrate degradation products such as furfural from pentoses and 5-HMF from hexoses; aldehydes such as glyceraldehyde and glycolaldehyde can also be obtained by retro-aldol condensation of D-glucose; lignin degradation products such as vanillin, syringaldehyde, and 4-hydroxybenzaldehyde; organic acids such as acetic acid from acetyl group, formic acid from xylose oxidation, and levulinic acid from glucose oxidation (X. Chen et al., 2009), (Saito et al., 2009). The operational temperatures that will promote degradation reactions depend on the lignocellulosic material and other operational parameters, as already discussed in previous

chapters (Chapters 3 and 4). In the case of AP, 5-HMF, furfural and phenolic compounds were detected at 170 ºC (Figures 5.5 and 5.6).

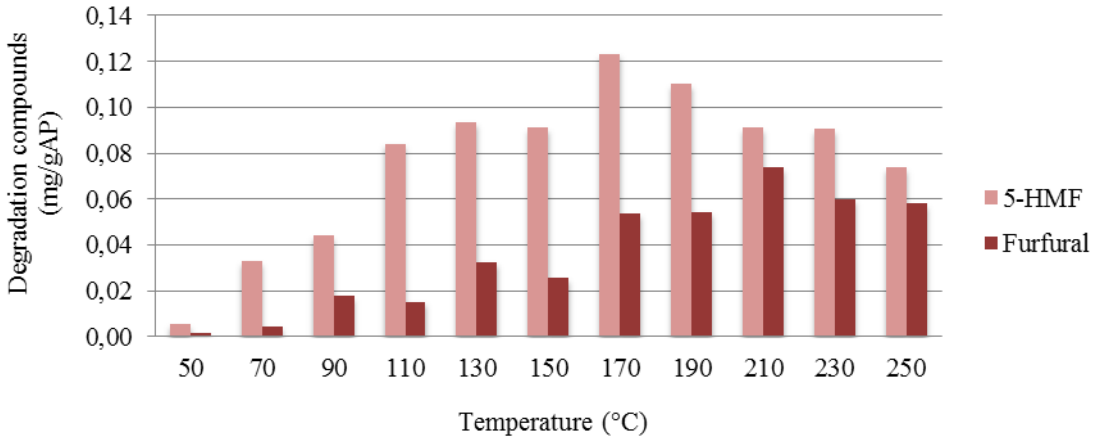


Figure 5.5: Effect of hydrolysis's temperature on the formation of degradation compounds from AP.

Previous studies already showed their potential to hinder yeast growth and so the importance of their identification and quantification (X. Chen et al., 2009). The amount of furfural was substantially lower than that of 5-HMF but a lower amount of pentoses is also present. Until 190 ºC, the amount of 5-HMF in the liquors sample is two times higher than the amount of furfural. This difference starts to be less significant at temperatures higher than 210 ºC.

Apple pomace HCW hydrolysis performed at temperatures higher than 190 ºC generates high amounts of both 5-HMF and furfural, which could have a negative impact in yeasts growth (Figure 5.5).

Total phenolic compounds were also monitored. Researchers have shown that a significant range of phenolic compounds (e.g. vanilic acid and vanillin) can generate inhibitory mechanisms on cell growth, albeit dependent on toxic concentration (Reed, G.; Nagodawithana, 1990). Although accurate analysis to obtain the phenolic compounds profile was not performed, this factor was taken into account by monitoring the phenolic compounds content.

From figure 5.6, an evident increase in the formation of phenolic compounds can be observed for temperatures higher than 150ºC suggesting that, operating at these temperatures, raises the probability of forming compounds that will later hinder yeast growth and lipid accumulation.

However, an important balance between avoiding phenolic or degradation compounds formation and high conversion must be considered in order to decrease the inhibitory effect not compromising the recovery of the necessary carbohydrates.

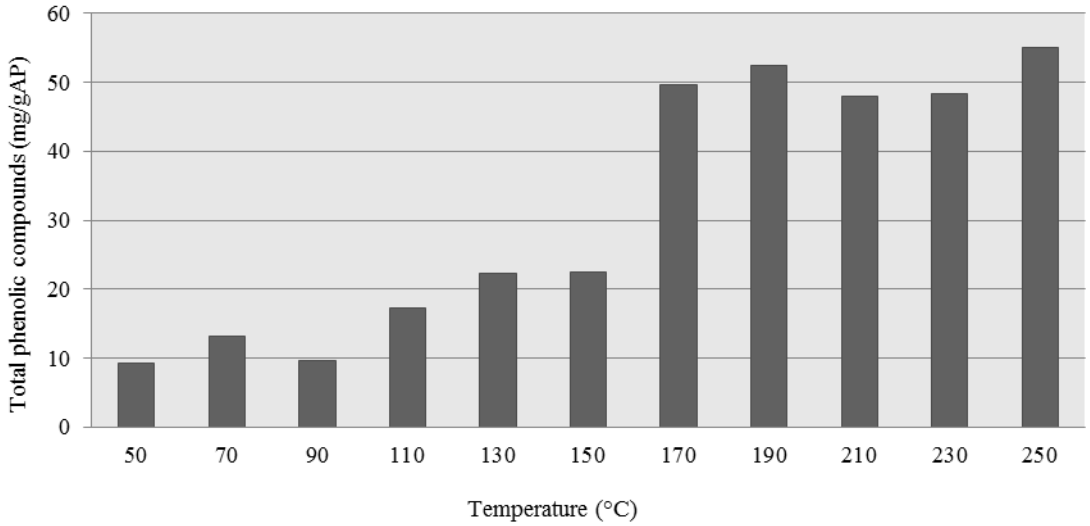


Figure 5.6: Effect of hydrolysis's temperature on the formation of phenolic compounds from AP.

Altogether, the results from figures 5.2 - 5.6 suggested that temperatures lower than 190 ºC (avoiding the presence of significant amounts of degradation compounds) and higher than 130 ºC (in order to obtain a high amount of WSC's with soluble oligosaccharides and monosaccharides) should be applied to apple pomace.

5.3.2. Apple pomace liquors as carbon source for oleaginous yeast growth

Five different oleaginous yeasts strains were pre-selected based on their lipids or carotenoids production activity (Beopoulos et al., 2009; Fall et al., 1984; Marova et al., 2012) matched with the ability to assimilate the major carbohydrates converted from the lignocellulosic biomass: *Rhodotorula babjevae*, *Rhodotorula toruloides, Rhodotorula glutinis, Rhodotorula mucilaginosa and Yarrowia lipolytica. Rhodotorula babjevae*, evaluated in previous chapter, was also included because of its good performance using the HCW treated GP liquors.

Among the five yeast strains presented, only *Yarrowia lipolytica* is a non-pigmented strain and fails to assimilate xylose and cellobiose (Table 5.2), it is, however, one of the most intensively studied non-conventional yeast with promising results regarding lipids accumulation (Beopoulos et al., 2008).

Table 5.2: Carbon sources assimilation profile for the five oleaginous yeasts strains (w-weak; d-delayed growth) (PYCC databank)(Leandro et al., 2009).

	Rhodotorula babjevae	***Rhodotorula toruloides***	***Rhodotorula glutinis***	***Rhodotorula mucilaginosa***	***Yarrowia lipolytica***
Arabinose	+	d	+	+	-
Xylose	+	+	+	+	-
Fructose	+	+	+	+	+
Galactose	+	+	+	w	d
Glucose	+	+	+	+	+
Cellobiose	+	-	D	+	-

Moreover, *Yarrowia lipolytica* is capable of degrading organic compounds, including aliphatic and aromatic hydrocarbons (Coelho et al., 2010), often produced during HCW hydrolysis systems.

Cellobiose is an important criterion once a significant portion of the carbohydrates recovered after HCW hydrolysis of AP are oligosaccharides (Figure 5.3). Cellobiose is a disaccharide, of two glucose units linked by a beta-1, 4-glycosidc bonds and is the building block for cellulose.

The potential of using AP liquors as carbon source to growth oleaginous yeasts was first evaluated using a blend of the liquors obtained at the 11 different temperatures. In order to obtain a significant mass amount, these liquors were lyophilized, blended and homogenized (called hydrolysate henceforth) (Figure 5.7) and used as single carbon source in minimum medium.

Figure 5.7: AP hydrolysates blend obtained from 11 different hydrolysis temperatures (A) and after homogenization (B).

The total amount of monosaccharides present in the blended hydrolysates was determined in order to achieve a final carbohydrates concentration in the growth medium of 12 g/L. This

corresponds to the sum of the five most abundant monosaccharides (glucose, fructose, galactose, xylose and arabinose), irrespective of whether other oligosaccharides, such as disaccharides, trisaccharides or higher, may also be present. A control assay was carried out with 12 g/L glucose as carbon source. Growth experiments were carried out in small-scale batches due to experimental set-up limitations, which result in small amounts of hydrolysates.

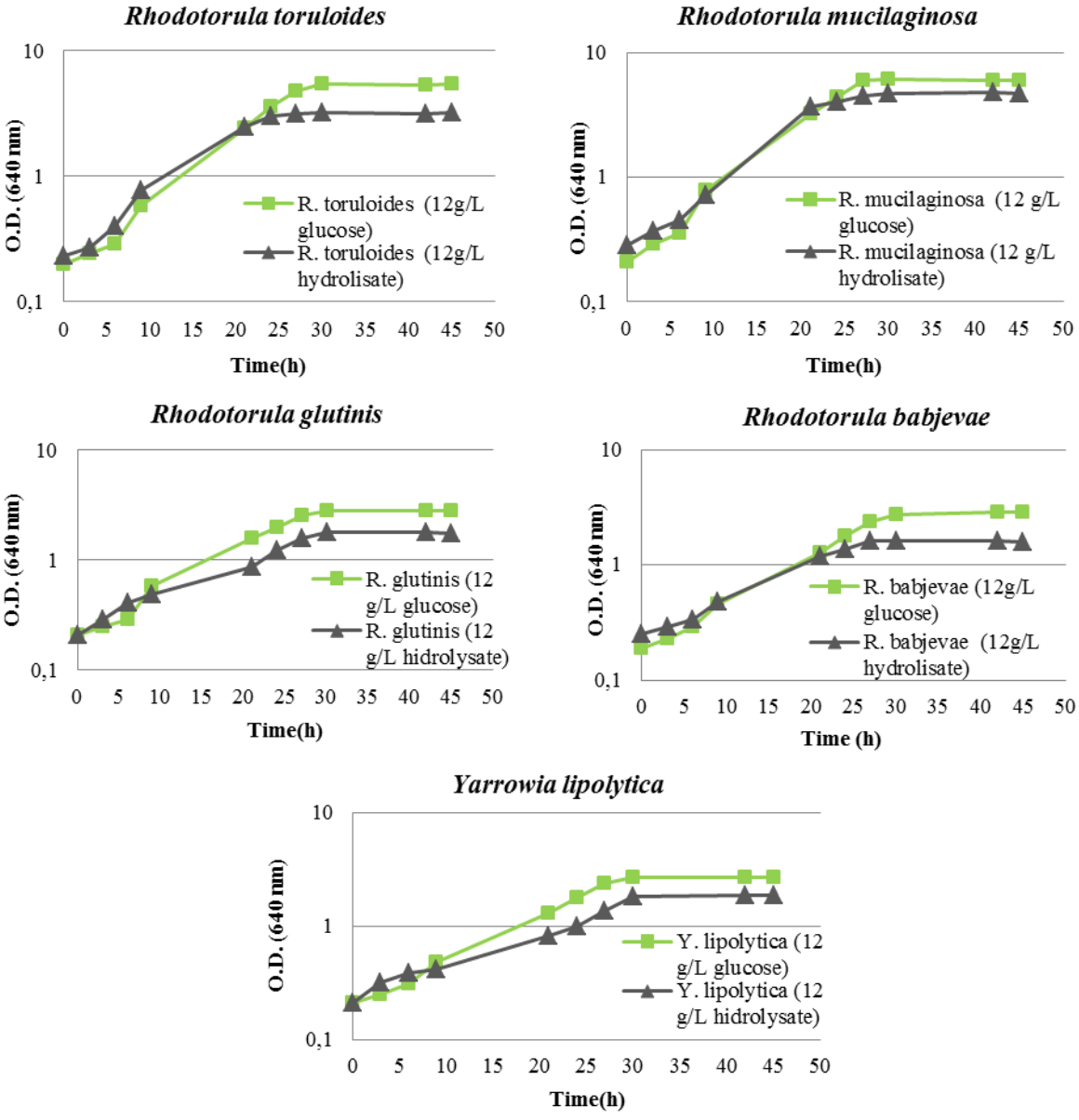


Figure 5.8: Growth curves for the five oleaginous yeasts strains on YNB and AP hydrolysates blend obtained between 50-250ºC. Yeast growth was performed at 25°C with constant agitation at 200 rpm in 1 mL batch culture.

As it can be observed from the growth curves represented in figure 5.8, all five selected strains grew on AP hydrolysates as the sole carbon source available. In standard medium (glucose),

yeast grows exponentially provided that the essential nutrients are available. After the exhaustion of one or more nutrients, cells enters the stationary phase, stop to proliferate and induces several specific physiological and biochemical changes, particularly they make and accumulate reserves. A similar behavior was observed for all strains both on glucose and AP hydrolysate (Figure 5.8). YNB medium is provided with essential nutrients in excess excluding the carbon source. Therefore, yeast growth will be compromised when the carbon source will be exhausted. Considering that the five yeasts strains are capable to assimilate all the five monosaccharides identified in the hydrolysates and the total available carbon amount is equal in both control and AP hydrolysates assays, other cause than the carbon source, must justify the difference between growth rates or biomass achievements as measured by the final OD. The medium with AP hydrolysates may contain amounts of furanic compounds such as 5-HMF (furfural presence is discharged as explained earlier) (Figure 5.5) or phenolic compounds (Figure 5.6) or other , which can justify the differences between growth behaviors.

The stress and inhibitors tolerance level is different for each microorganism depending from genera, species or even strain but a screening using *Rhodotorula glutinis* and *Rhodotorula toruloides* were made testing their capability to growth and accumulate lipids in the presence 0,5 to 1 g/L of several lignocellulosic material degradation compounds (X. Chen et al., 2009). Acetic acid, formic acid, furfural, and vanillin were classified as strong growth inhibitors, while levulinic acid, 5-HMF, and hydroxybenzaldehyde were considered as relatively weak ones. Among organic acids, acetic acid turned out to be the strongest inhibitor at a concentration of 5 g/L. It is important to stress the fact that acetic acid is one inevitable by-product from the hemicellulose hydrolysis and, depending on the treatment conditions, the concentration of acetic acid may reach values as high as 10–20 g/L (Kamm, B.; Gruber, P.R.; Kamm, 2008). Several other studies already demonstrated the potential inhibitory effect of some of the degradation compounds mentioned above. Growth of specific strains of *Saccharomyces cerevisiae* and *Pichia stipitis* demonstrated to be dose-dependent at concentrations from 10 to 120 mM for furfural and HMF inhibition, respectively (Liu et al., 2004). Other study performing a screening for the tolerance of several lignocellulose degradation compounds to *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Rhodotorula toruloides* and *Lipomyces starkeyi* demonstrated that, from furan compounds, furfural was found to be the strongest inhibitor; 5-HMF was shown to be a mild inhibitor and vanillin, a phenol derivative, to be a strong inhibitor similar to acetic acid and furfural potential (X. Chen et al., 2009).

Another study considering the biomass hydrolysis by-products effect on *Rhodotorula toruloides* growth and lipid accumulation performance concluded that acetate, 5-HMF and syringaldehyde were compounds with a weak inhibitory effect; p-hydroxybenzaldehyde and vanillin were toxic at a concentration above 10 mM; and furfural and its derivatives furfuryl alcohol and furoic acid inhibited cell growth by 45% at around 1 mM. (Hu et al., 2009).

The results from figure 5.8 indicate that the strains of *Rhodotorula mucilaginosa* and *Rhodotorula toruloides* seem better adapted to assimilate the carbon from AP liquors, attaining the closest values to the standard medium (either maximum OD values or growth rate). The comparison of the growth curves (Figure 5.9) also shown that the same two *strains* are the most interesting.

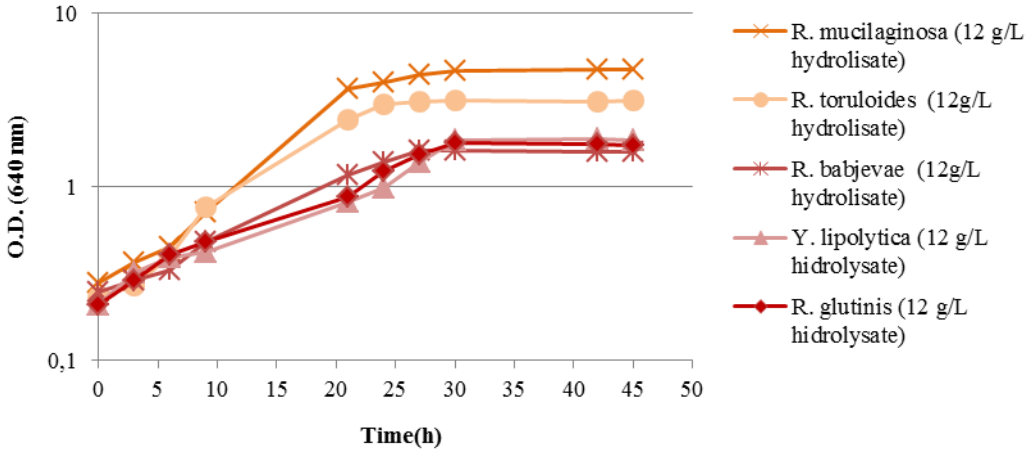


Figure 5.9: Comparison of yeast growth on AP hydrolysates.

In conclusion, these results have shown the potential of apple pomace HCW treated as an alternative carbon source for sustaining yeast growth, as was already shown for GP pomace (chapter 4), representing a possible cost reduction in industrial terms due to the implementation of a inexpensive lignocellulosic raw material as carbon source.

5.3.3. Apple pomace hydrolysates potential for lipid accumulation by yeasts

In the previous section, the viability of using the hydrolysates as single carbon source to growth yeasts was already demonstrated. The second step is to evaluate AP hydrolysates potential not only to grow yeasts but also to be used for lipids accumulation, for that purpose, a medium

described by *Zhao et al., 2008* to promote lipids accumulation was used as described under Materials and Methods of this chapter.

Several studies have shown that a high carbon to nitrogen (C/N) ratio, around 90, is a basic requirement for lipids accumulation (Chang, Y.; Chang, K.; Jang, H.; Hsu, 2011). As previously referred, when yeast enters the stationary phase of growth, it channeled the metabolism towards the production of reserves. If an essential nutrient, such as nitrogen, is exhausted from the medium while plenty of carbon is still available, oleaginous yeast channeled them to the production of lipids. Therefore, oleaginous potential is extremely affected by the C/N ratio of the culture as well as other factors like aeration, inorganic salts, among others (Moreton, 1988). For these reasons, a previously designed medium to promote lipids was used.

The culture medium contains 70 g/L of AP hydrolysate blend collected from 50-250ºC as the only carbon source available against a control medium containing the same quantity in glucose. The medium using the AP hydrolysate contains the same equivalent quantity in monosaccharides that the medium containing only glucose. As it can be observed from figure 5.10, all the strains had a good growth performance. The growth performed in the medium containing glucose resulted in higher OD's for the five yeasts.

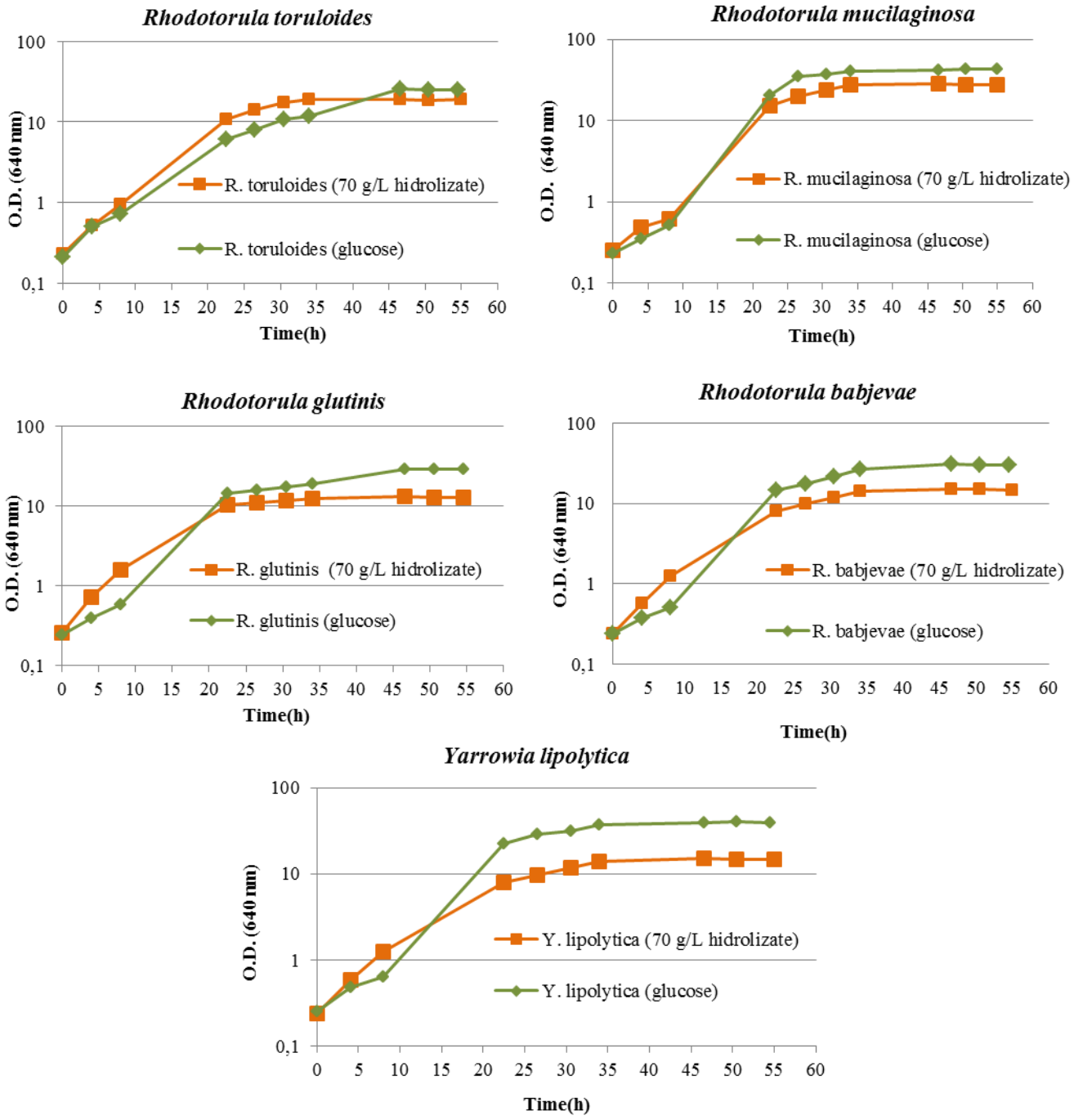


Figure 5.10: Growth curves of five oleaginous yeasts strains using apple pomace hydrolysates for lipid accumulation.

By using this specific designed medium for lipids accumulation, yeasts growth will be limited by the amount of nitrogen available in the medium. AP composition contains an average of 2.74% of proteins (table 5.1), which could result in an extra source of nitrogen. On the other hand, HCW hydrolysis can lead to protein degradation (Zhang et al., 2013). Therefore, the somewhat weaker performance for yeast growth when using AP hydrolysates might be explained by some inhibitory effect of the hydrolysates already discussed in the previous section.

The difference between the total cell biomass using hydrolysates and the control was less significant for *Rhodotorula mucilaginosa* and *Rhodotorula toruloides*. This diversity in growth yeasts performances could be related with the sensibility of each strain to the presence of inhibitors.

In figure 5.11 it is possible to compare the growth curves for the five yeasts. Similar to the results presented in the previous section, *Rhodotorula mucilaginosa* presented the best growth performance in terms of AP hydrolysates assimilation represented by the highest cell biomass concentration achievement.

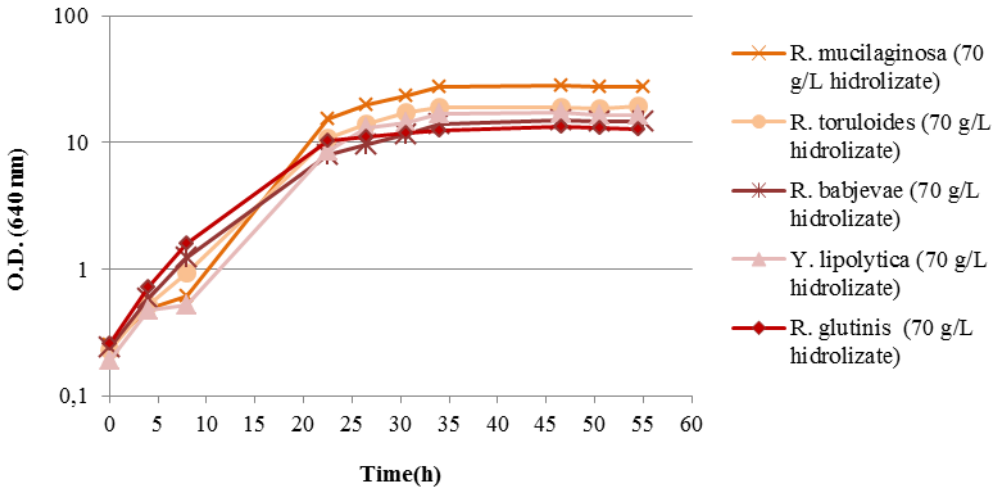


Figure 5.11: Comparison between growth curves from five yeasts using apple pomace hydrolysates for lipid accumulation.

Lipid accumulation by yeast cells was evaluated by fluorescence microscopy (Figure 5.12, 5.13, 5.14, 5.15, 5.16). A quantitative lipid accumulation analysis was not possible due to the low scale in which yeasts cells culture was performed thus a semi-quantitative lipid accumulation analysis was performed counting the number of cells containing lipid droplets in a total of 10 cells randomly selected from images captured from integrated fluorescence microscope software. The following figures are representative images from lipids droplets (white dots within the cell) present in different strains, observed with 100x objective and detailed cell expanded 70%. This was a rough image analysis that, nevertheless, allowed for checking the lipids droplets and a first comparison between strains.

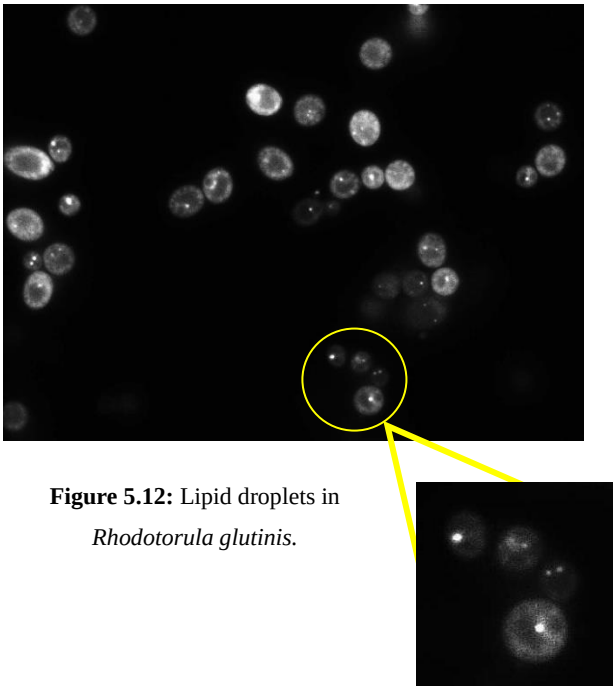


Figure 5.12: Lipid droplets in *Rhodotorula glutinis.*

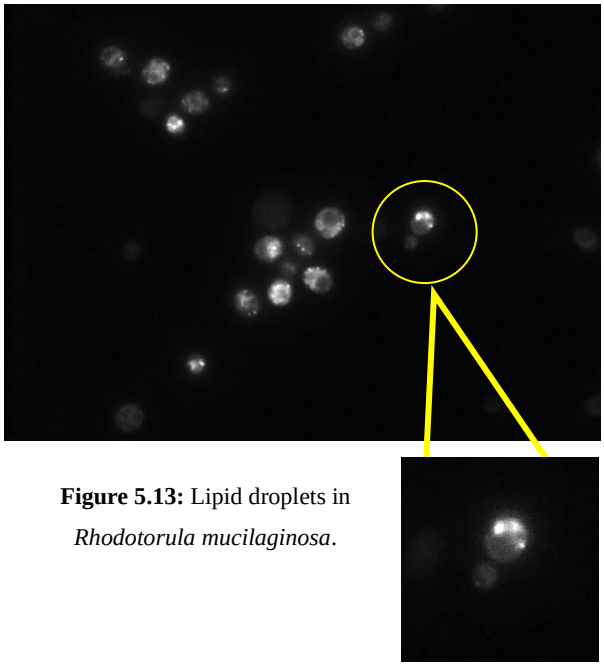


Figure 5.13: Lipid droplets in *Rhodotorula mucilaginosa*.

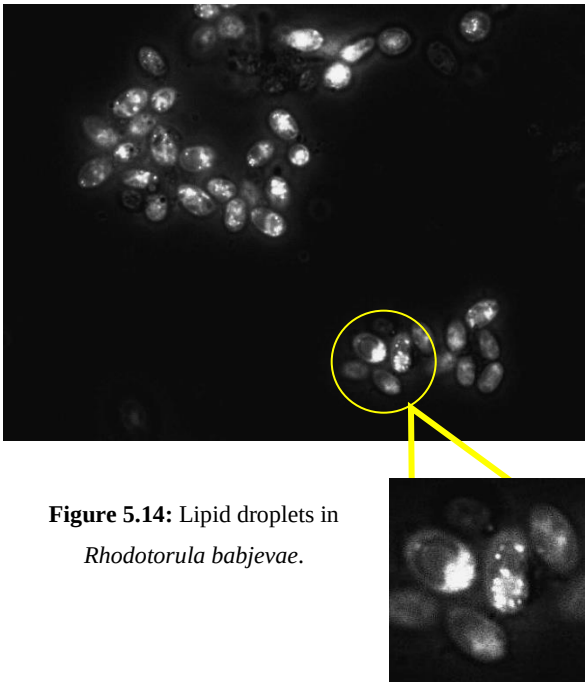


Figure 5.14: Lipid droplets in *Rhodotorula babjevae*.

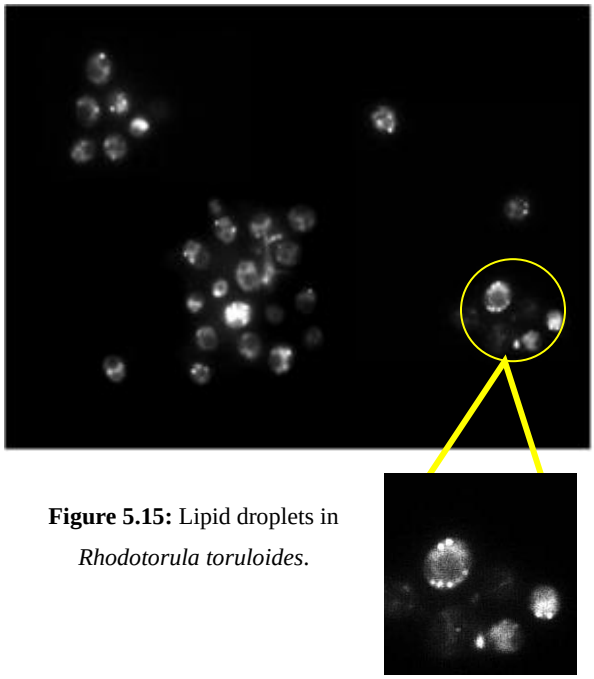


Figure 5.15: Lipid droplets in *Rhodotorula toruloides*.

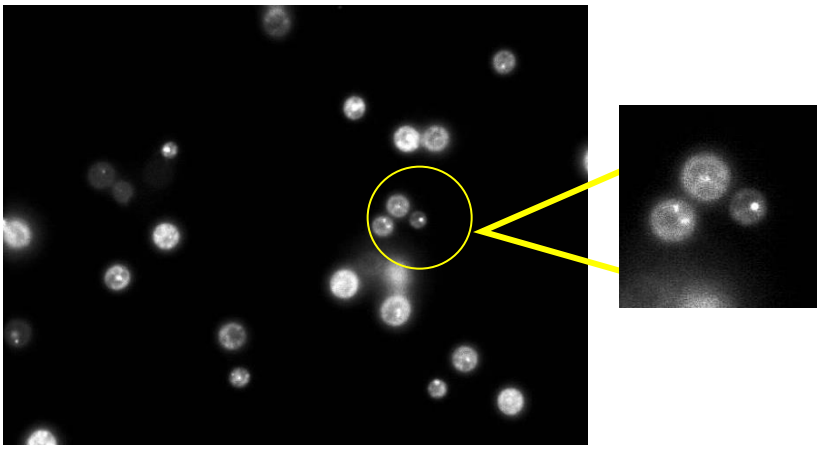


Figure 5.16: Lipids droplets in *Yarrowia lipolytica*.

Similar results were obtained for the five strains in study with approximately 90% of the cells showing lipid droplets with no remarkable differences. The small scale used did not allow an accurate conclusion about lipid accumulation for each strain mainly because it was not possible to perform their extraction and quantification by HPLC. Therefore, cells culture scale-up is necessary in order to obtain a significant biomass amount for further quantitative analysis.

Based on these preliminary results, it is possible to conclude that the strain that showed better performance when grow in AP hydrolysate was *Rhodotorula mucilaginosa* and it was selected for the following experimental studies.

5.3.4. HCW treatment of AP at selected temperatures to obtain hydrolysates for *Rhodotorula mucilaginosa* growth

Considering the results presented under sections 5.3.1 to 5.3.3 and the balance between the amount of monosaccharides retrieved and its degradation, it can be concluded that the best temperature range is 130 - 190 °C. Therefore, four different assays were performed at the following 4 selected temperatures: 130 °C, 150 °C, 170 °C and 190 °C. Each assay was performed during 20 min after reaching the selected temperature. All liquors were collected in a single sample.

Similar to the previous experiment, all liquors collected were analyzed in terms of WSC's, total carbohydrates and oligosaccharides, monosaccharides, degradation compounds and total phenolic contents.

WSC's recovery is not significantly different between temperatures to perform a selection only based on these results (Figure 5.17).

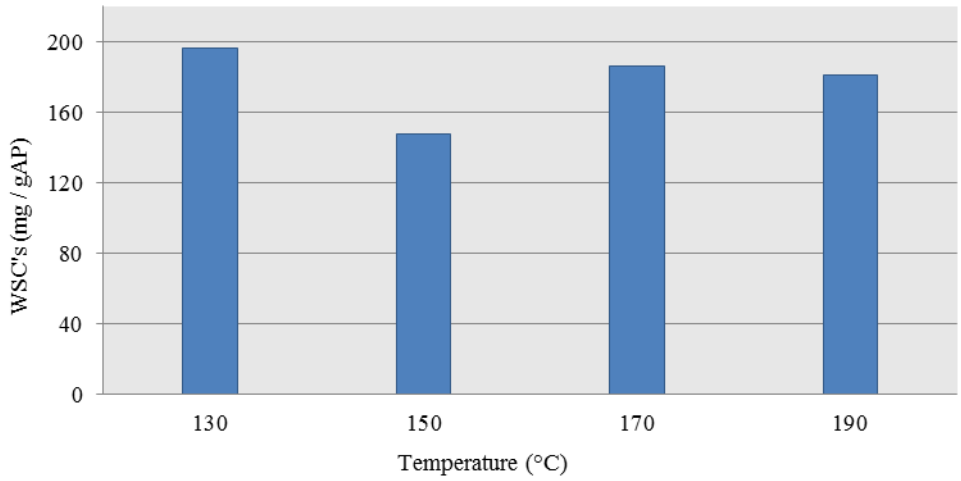


Figure 5.17: WSC's recovery for the 4 different temperatures.

The highest amount of total carbohydrates was obtained for the experiments performed at 130 °C in which 32% are solubilized as monosaccharides (Figure 5.18 and Table 5.3).

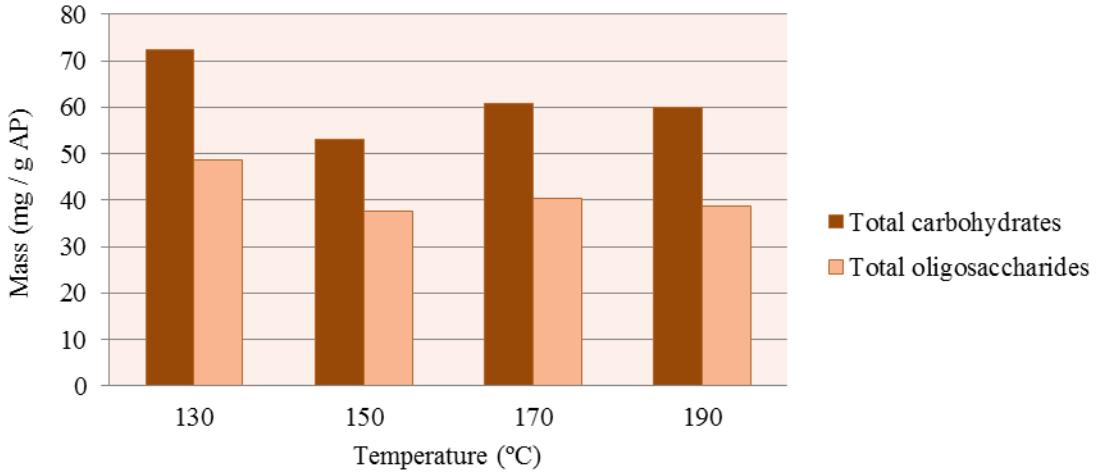


Figure 5.18: Total carbohydrates and oligosaccharides recovery for each of the four selected HCW hydrolysis temperatures.

Table 5.3: Oligosaccharides and monosaccharides content in the liquors obtained at 4 different temperatures.

Temperature (°C)	Total oligosaccharides (mg/g AP)	Glucose (mg/g AP)	Xylose + Galactose (mg/g AP)	Fructose + Arabinose (mg/g AP)
130	49	8	0	15
150	38	6	1	9
170	40	9	1	10
190	39	9	1	11

At temperatures above 130 ºC, a decrease in total oligosaccharides (table 5.3), an increase in the amount of 5-HMF (figure 5.19) as well as an increase in the amount of phenolic compounds present in the liquors (figure 5.20) were observed which is according with the temperature effect on hydrolysis extension already mentioned under section 5.3.1.

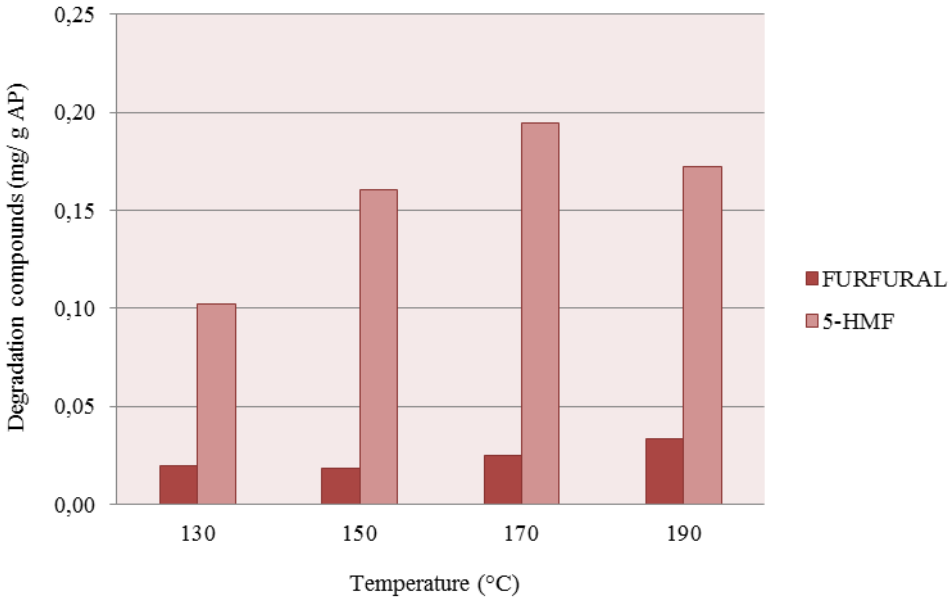


Figure 5.19: Degradation compounds formation for the 4 different temperatures.

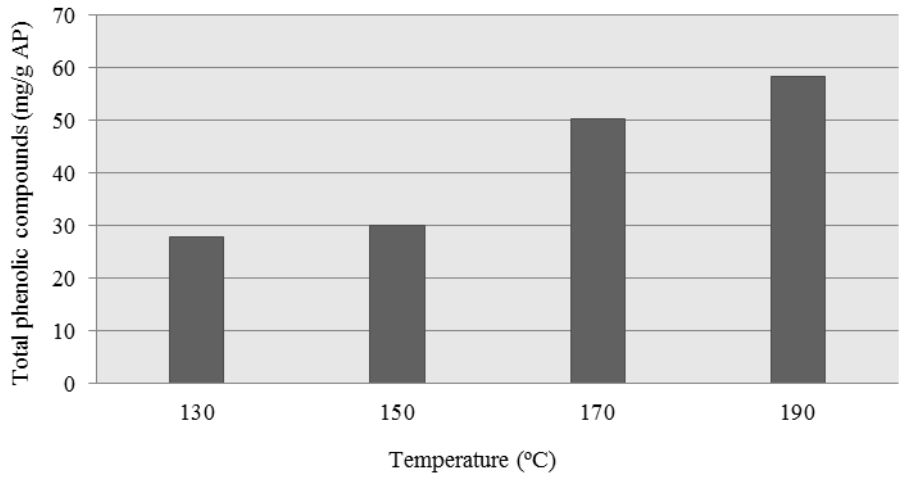


Figure 5.20: Phenolic compounds formation for 4 different temperatures.

5.3.5. Apple pomace HCW hydrolysis temperature optimization for *Rhodotorula mucilaginosa* optimal growth and lipid accumulation

Temperature optimization was performed by selecting HCW temperature that leads to AP liquors with a high amount of oligosaccharides and monosaccharides and, at the same time that leads to a reduction of degradation and phenolic compounds. Additionally, the liquor at the selected temperature must also promote the best growth performance as well as the highest lipid accumulation by *Rhodotorula mucilaginosa*. Therefore, the same strategy applied in the previous section was used to evaluate *Rhodotorula mucilaginosa* lipid accumulation at the liquors collected at 130 ° C, 150 ° C, 170 ° C and 190 ° C.

Growth media were prepared with freeze-dried powders with total amount of monosaccharides kept at 70g/L (in carbon source). Although the four growth medium had the same sugar amount, the proportion between monosaccharaides or other degradation compounds might vary among liquors.

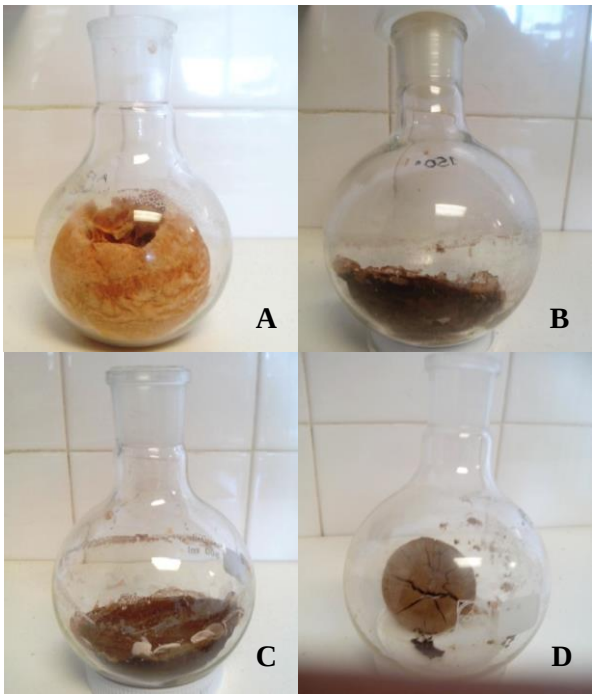


Figure 5.21: Apple pomace hydrolysates obtained from HCW hydrolysis performed at 130 ºC (A), 150 ºC (B), 170 ºC (C) and 190 ºC (D).

The results presented in figure 5.22, as previously seen in figure 5.8 and 5.10, show that higher biomass amounts were obtained for the growth performed using glucose as the sole carbon source. Interestingly is the difference between hydrolysates. Undoubtedly, the hydrolysis at 130 ºC provides the best substrate to *Rhodotorula mucilaginosa* (OD_{640nm} =26.5 at 50h), approximately, two times higher than the biomass obtained for others hydrolysates ($OD_{150ºC,640nm}$=12; $OD_{170ºC,640nm}$=15; $OD_{190ºC,640nm}$=14).

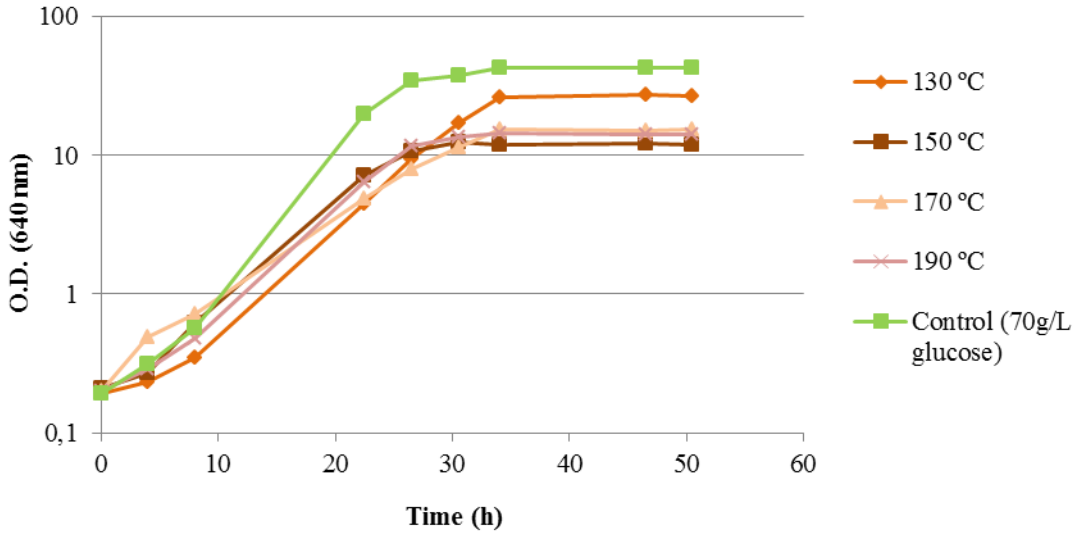


Figure 5.22: Growth curves of *Rhodotorula mucilaginosa* using apple pomace hydrolysates obtained at 130 ºC, 150 ºC, 170 ºC and 190 ºC.

Lipid accumulation analysis was also performed in the same conditions as before. As expected, lipid droplets were observed for all the temperatures for 90% of the cells (figures 5.23, 5.24, 5.25 and 5.26).

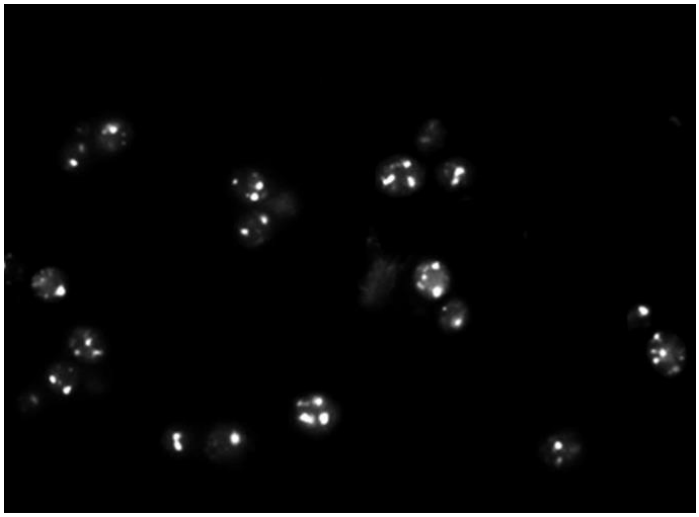


Figure 5.23: Lipid droplets observed in *Rhodotorula mucilaginosa* after growth on apple pomace hydrolysate obtained at 130 ºC.

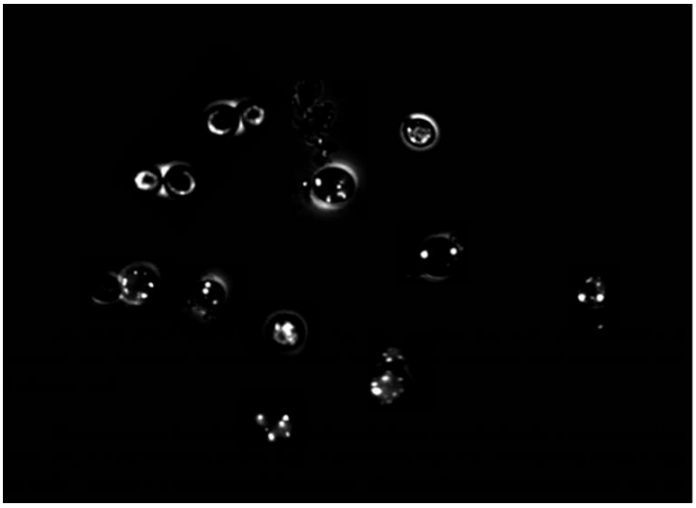


Figure 5.24: Lipid droplets observed in *Rhodotorula mucilaginosa* after growth on apple pomace hydrolysate obtained at 150 ºC.

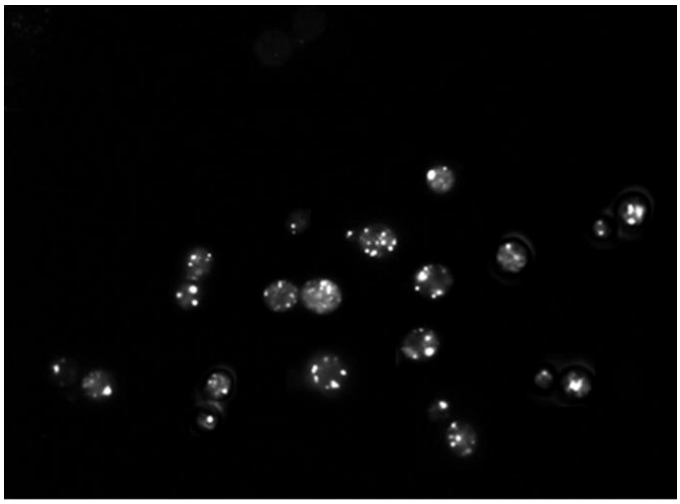


Figure 5.25: Lipid droplets observed in *Rhodotorula mucilaginosa* after growth on apple pomace hydrolysate obtained at 170 ºC.

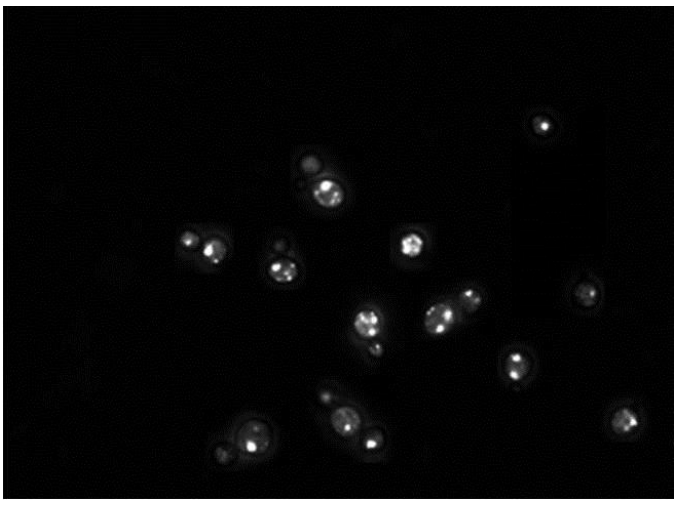


Figure 5.26: Lipid droplets observed in *Rhodotorula mucilaginosa* after growth on apple pomace hydrolysate obtained at 190 ºC.

Analyzing the results obtained from *Rhodotorula mucilaginosa* growth performance in different AP hydrolysates obtained at 130°C, 150°C, 170°C and 190°C and respective lipid accumulation occurred in each assay, 130 ºC apparently provide the most interesting conditions. Knowing that the quantity of biomass directly influences the amount of lipids that can be recover, we selected the AP hydrolysate obtained by hydrolysis performed at 130°C for an experiment in a higher scale that increase the possibility of extraction and quantification of lipids and or carotenoids.
It is also important to notice that when a scale up trial is designed, the economic viability of the process must be analyzed and higher temperatures leads to higher energy costs. Therefore, the selection of using AP hydrolysate obtained from HCW hydrolysis performed at 130°C for further study development is also an economically advantageous option.

5.3.6. Lipid accumulation by *Rhodotorula mucilaginosa* using AP hydrolysate at 130 ºC as single carbon source – Scale up

A preliminary scale-up was performed using a growth culture volume 10 times higher (10 ml) than the growth experiments performed previously. Increasing the operation scale, it is expected to obtain a sufficient biomass amount, which allows a quantitative analysis of lipids and carotenoids produced by *Rhodotorula mucilaginosa*. Growth culture medium was prepared as the previous, but using a volume 10 times higher (10 ml) than before.

The results (Figure 5.27) reproduced what was already observed for the smaller volume (Figure 5.22) which is truly satisfactory and demonstrate AP hydrolysates potential to be used as single carbon source for oleaginous yeasts growth since *Rhodotorula mucilaginosa* was able to achieve already 62% of the total biomass yield obtained when using glucose as single carbon source without performing any growth optimization conditions.

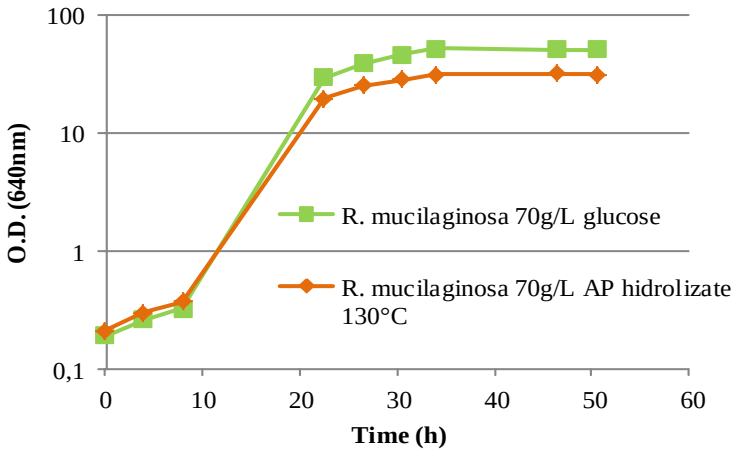


Figure 5.27: *Rhodotorula mucilaginosa* growth curve in the presence of apple pomace hydrolysate obtained at 130 ºC and glucose (scale-up).

Regarding lipid accumulation, a noteworthy different accumulation pattern was observed. In the latter experiment lipid droplets were small and in large quantity within a single cell (Figure 5.23), whereas the current cells exhibit only one droplet but apparently larger (Figure 5.28). This observation is referred for the results obtained using AP hydrolysates as well as for the control. Moreover, no significant difference between lipids accumulation using AP hydrolysates and glucose was observed which represents also a very important result.

Lipid accumulation in oleaginous yeast is known to be influenced by growth conditions, especially the carbon source, the nitrogen source, minerals and aeration (Zhao et al., 2008).The new conditions can justify the difference in *Rhodotorula mucilaginosa* lipid droplets. The higher volume increase as well as the use of an Erlenmeyer flask instead of a tube test could have promoted a more efficient aeration during growth. It is very important to explore, as future work, growth conditions parameters that might result in lipid accumulation maximization by oleaginous yeasts.

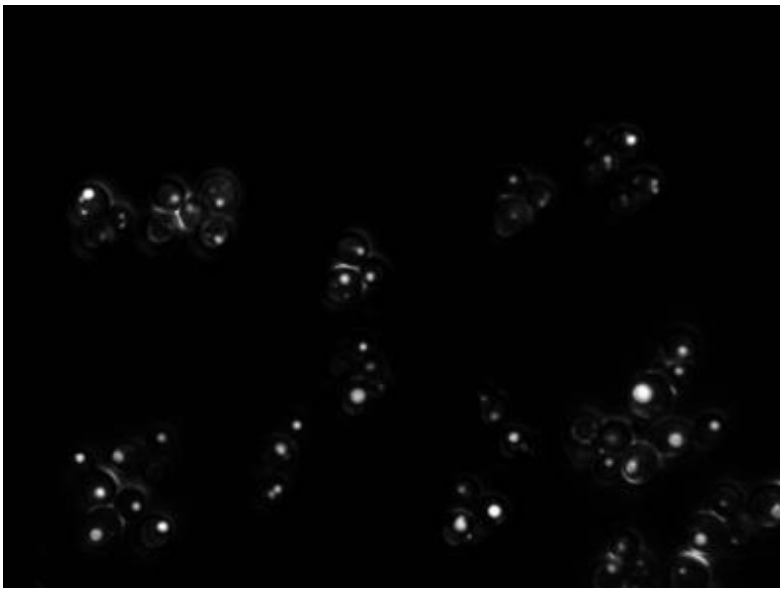


Figure 5.28: Lipid droplets in *Rhodotorula mucilaginosa* after grown on apple pomace hydrolysate obtained at 130 ºC.

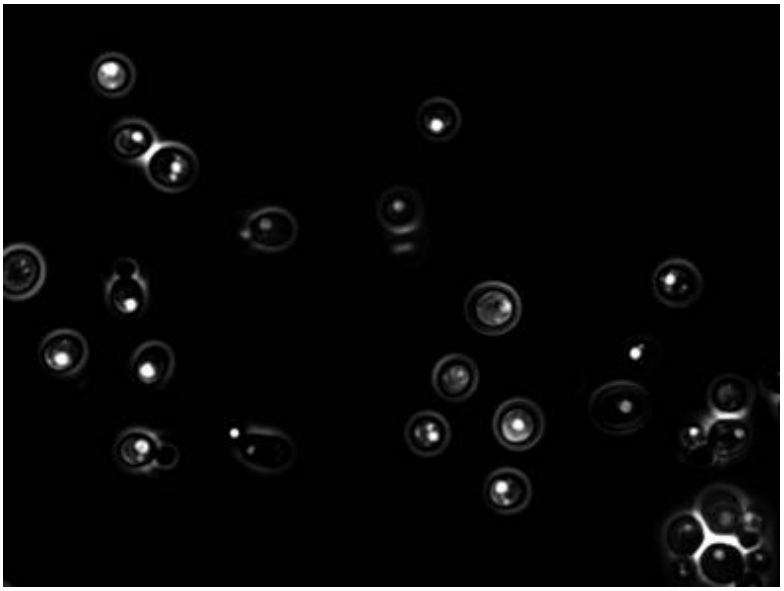


Figure 5.29: Lipid droplets in *Rhodotorula mucilaginosa* after grown on glucose (control assay).

Recalling that the most commonly used carbon source for single cell oil production is glucose, it is very important to find an alternative low cost raw material capable to promote oleaginous yeasts growth and lipid accumulation in order to reduce the cost of single cell oil production. From the preliminary results presented above, even without any growth conditions optimization, it is possible to conclude that AP hydrolysates have a great potential to be used as alternative carbon source.

5.3.7. *Rhodotorula mucilaginosa* carotenoids production

Lipids and carotenoids produced from *Rhodotorula mucilaginosa* from the later experiment were extracted using hexane as organic solvent under agitation during 16h (Figure 5.30). HPLC analysis was carried out to determine the content of β-carotene in the extracts. The β –carotene, which confers to many pigmented fruits and vegetables its color , is one of the most studied carotenoids and has an important role in nutrition and human metabolism since plays an important physiological role as a precursor of vitamin A (retinol) and retinoic acid (Olson, 1960).

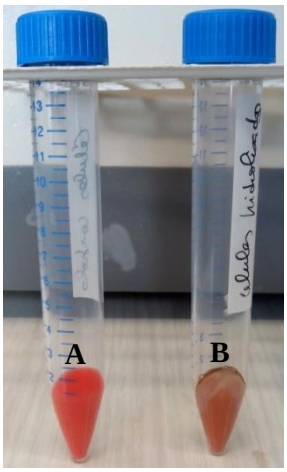


Figure 5.30: Biomass obtained for carotenoids quantification. A- Control assay on glucose, B- Cells grown on apple pomace hydrolysate.

The results obtained after organic extraction from cell-dried biomass are presented in table 5.4. In control assay, 286 mg of cell dry weight was obtained, higher than the 197 mg of cell dry weight obtained on the AP hydrolysate corroborating the 60-70% higher value for the OD mentioned earlier. The organic extract contains all the components soluble in hexane, mainly lipids and carotenoids, accumulated by yeasts during growth. Cells in the control assay were able to accumulate lipids and carotenoids corresponding to 24.2% in a dry biomass base against 11.3% from cells that grew on AP hydrolysate. Cells accumulation performance using AP hydrolysates was lower (less than 50%) comparing with the accumulation in cells that used glucose.

Table 5.4: Total carotenoids and β-carotene production by *Rhodotorula mucilaginosa*.

	Control	**Growth on AP hydrolyzate**
Dried cell biomass	286 mg	197 mg
Organic extract	**69.27 mg** 24.2% (mg extract/mg dry biomass)	**22.15 mg** 11.3%(mg extract/mg dry biomass)
Total carotenoids	**9.31 µg/g dry biomass**	**7.02 µg /g dry biomass**
β-carotene	**5,11 µg /mg** 55% (mg β-carotene / mg $T_{carotenoids}$)	**2,31 µg /mg** 33% (mg β-carotene / mg $T_{carotenoids}$)

According to the literature, *Rhodotorula mucilaginosa* is able to accumulate carotenoids achieving values of 152 µg/g dry cell (Maldonade et al., 2012). Using only AP hydrolysates as single carbon source, *Rhodotorula mucilaginosa* was able to accumulate 7,02 µg/g dry biomass, which represents 75% of the potential observed using only glucose (9,31 µg/g dry biomass) but corresponds to 5% of its maximum potential for carotenoids accumulation. Several studies have shown that the culture medium (C/N ratio, minerals composition, etc.) and experimental conditions such as temperature, pH and oxygen can influence yeasts biomass accumulation and carotenoids production (Naghavi et al., 2014) and it was already possible to prove *Rhodotorula mucilaginosa* lipid and carotenoids accumulation potential by using AP hydrolysates with an similar performance when compared with the control essay.

Considering only the total carotenoids analysis obtained by UV-vis spectrometry, the extract obtained in the control assay is richer in β-carotene (55% of total carotenoids is β-carotene) than the extract using biomass grown in AP hydrolysate (33% of total carotenoids is β-carotene) (Table 5.4). Optimization growth experiments need to be performed in order to increase the amount of lipids and carotenoids accumulated by the cells.

5.4. Conclusions

HCW represents an environmentally friendly solvent and an attractive reaction medium for several applications. The main goal of the present work was to evaluate the potential of applying HCW to hydrolyze AP to be used as a sole carbon source substrate to growth oleaginous yeast able to accumulate lipids and carotenoids.

A simple strategy was applied to evaluate HCW hydrolysis temperature effect in the solubilized material recovered. The amount of WSC's, total carbohydrates and monosaccharides recovered were higher for temperatures between 130 °C and 190 °C. The reason is the benefit/quality ratio between hydrolysis extension and biomass decomposition during the process. The conclusion was that a low hydrolysis temperature is needed in order to recover the maximum carbon structures solubilized, avoiding the presence of degradation compounds and turning the process economically more competitive. A three stage AP valorization process is proposed where (1) directly soluble monosaccharides are extracted using HCW at a low temperature followed (2) by a HCW hydrolysis performed at 130 ºC obtaining a liquor rich in oligosaccharides which can be further (3) hydrolyzed using enzymes which would hydrolyze more easily and in less time the remaining structure.

Five different oleaginous yeasts strains were tested to demonstrate the suitability of the AP hydrolysates material as carbon source for growth and for lipids and carotenoids production. The best growth performance was achieved with hydrolysates collected at 130 °C by *Rhodotorula mucilaginosa*. However, comparing growth performances when using AP hydrolysates with the control assay using only glucose, it is possible to conclude that AP hydrolysates had a potential inhibitory effect on oleaginous yeasts growth. More experiments need to be performed in order to explore AP hydrolysates chemical composition profile, mainly to identify the presence of inhibitors and toxic compounds that could reduce yeast growth performance and lipids and carotenoids accumulation. All five strains accumulated lipids after using AP hydrolysates as carbon source and *Rhodotorula mucilaginosa* was able to use the hydrolysate to accumulate carotenoids with similar performance when comparing with the control assay.

Several operational parameters can also be optimized to achieve yeast best performances in terms of growth and lipids and carotenoids accumulation.

6

Continuous enzymatic production of biodiesel from virgin and waste sunflower oil in supercritical carbon dioxide

The work presented in this chapter was published as Rodrigues, A. R., Paiva, A., Gomes, M., Simões, P., & Barreiros, S. (2011). Continuous enzymatic production of biodiesel from virgin and waste sunflower oil in supercritical carbon dioxide. *The Journal of Supercritical Fluids*, *56*(3), 259–264. http://doi.org/10.1016/j.supflu.2010.10.031.

Lipids produced by oleaginous yeasts can be used as an alternative lipid source for biodiesel, presently produced from palm oil, rapeseed or soybean derived lipids (Santamauro et al., 2014). However, it was not possible to study the potential of using directly the oleaginous yeast oil obtained from the study presented in the last chapter due to the low amounts recovered. Therefore, other alternative oil sources were considered in order to develop the proof of concept. The process development was performed considering two different sources: edible sunflower oil and waste cooking oil.

6.1 Introduction

Economic development has been accompanied by growing energy needs (Silver, 2008). At the current rate of increase in population in developing countries, it is estimated that existing oil reserves will be exhausted in this century. Oil consumption worldwide is expected to increase until the supply is no longer sufficient to meet the demand (British Petroleum, 2009). The use of biofuels, such as biodiesel, can help mitigate this problem.

Although biodiesel is considered as a viable alternative to petrol diesel (Demirbaş, 1998), the continuing controversy around the "greenness" and economic advantages of biodiesel makes its industrial application difficult. Indeed the conventional biodiesel production process via methanolysis of cooking oil with inexpensive alkaline catalysts, such as sodium hydroxide, raises environmental concerns due to the large amount of waste produced (Demirbaş, 1998; Fukuda et al., 2001; Kraeczyk, 1996; Robles-Medina et al., 2009; Soumanou and Bornscheuer, 2009; Vicente et al., 2004). Enzymes, on the other hand, are biological catalysts that work under mild reaction conditions and are more selective, leading to less waste (Fukuda et al., 2001; Robles-Medina et al., 2009; Soumanou and Bornscheuer, 2009; Vicente et al., 2004). Additionally, enzymes represent an environmentally friendly alternative to chemical catalysts.
Biodiesel production can further conform to environmental concerns if volatile, toxic, and flammable organic solvents are avoided and replaced with supercritical carbon dioxide ($ScCO_2$) (Wen et al., 2009). CO_2 is a very attractive solvent due to its nonflammability, low toxicity and high availability (Matsuda et al., 2005b). One advantage of using $ScCO_2$ is its ability to diffuse through solid matrices, such as immobilized enzyme preparations, as well as the improved diffusivities of the dissolved components in the reaction media (Matsuda et al., 2005b). The solvation ability of $ScCO_2$ can be easily modified by adjusting temperature and pressure conditions (Reverchon and Marco, 2006). Through the manipulation of T and P, the solubility of a given substance in $ScCO_2$ can increase, or decrease to the point of precipitation (Jackson and King, 1996). This greatly simplifies the downstream cleanup process.

The major challenges for biodiesel production are cost and limited availability of fat and oil resources. The cost of raw materials accounts for 60 to 75 % of the total cost of biodiesel fuel against 40 to 25 % for processing costs (Haas et al., 2006). The use of waste cooking oil (WCO), which is a residue without commercial value and whose disposal is problematic (Kulkarni and Dalai, 2006), can have a significant effect on the economy and environmental impact of biodiesel production. Assuming that the transport and pretreatment of WCO are the only additional costs involved in the production of biodiesel from WCO, the effect of the price of raw material in the biodiesel production costs can be reduced by as much as 50 % (Demirbas, 2009b).

In the present work, a $ScCO_2$-based continuous, integrated process, comprising the enzymatic synthesis of biodiesel via a methanolysis reaction followed by downstream separation is described, using as starting material virgin and waste cooking sunflower oil. Reaction parameters were optimized firstly for virgin oil. Reactions were carried out at conditions favouring the solubility of the oil in $ScCO_2$. The viability of using $ScCO_2$ as a solvent for the fractionation of the reaction products, namely biodiesel, glycerol, methanol and mono-, di- and triacylglycerols, was assessed in a separation section comprising two high pressure separators. The process was replicated with WCO. In this case, experiments were performed using either Novozym 435 or Lipozyme TL IM alone, as well as a combination of both enzymes (Li et al., 2006).

6.2. Materials and Methods

6.2.1. Materials

The edible sunflower oil used in the experiments was from Fula®. Methanol and *n*-hexane of chromatographic grade (99.9 % and 97 % purity, respectively) were supplied by Sigma-Aldrich. Carbon dioxide (99.98 % purity) was from Air Liquide. The standard for the determination of FAME by gas chromatography (GC) was from Sigma-Aldrich. Heptadecane from Fluka was used as internal standard. Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on an acrylic resin) and Novozym 435 (*Candida antarctica* lipase B immobilized on a macroporous resin) were a gift from Novozymes A/S, Bagsvaerd, Denmark. Waste cooking sunflower oil was provided by a local restaurant at the Faculdade de Ciências e Tecnologia *campus*. Before it was fed to the reactor, WCO underwent a simple gravity filtration to remove food residues.

6.2.2. High-pressure apparatus

A schematic diagram of the apparatus is shown in Figure 6.1.

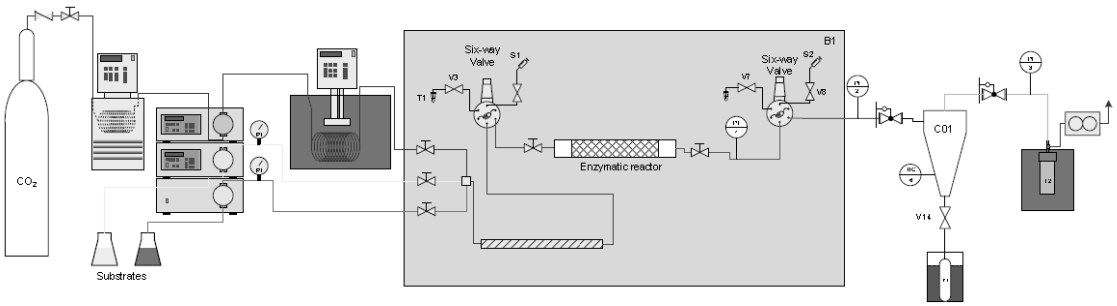


Figure 6.1: Experimental apparatus. B, temperature control bath; P, liquid pump; T, sample trap; V, valve; S, syringe; TI, temperature indicator; PI, pressure indicator; C0, high pressure cyclone separator, FM, flow meter; BPR, back pressure regulator valve.

The apparatus for continuous biodiesel production consists of two main sections, namely a reaction section comprising a high pressure packed-bed enzymatic tubular stainless steel reactor, and a separation section.

CO_2 is liquefied in a refrigerator containing a water/ ethylene glycol solution, before being pumped with an HPLC pump (maximum flow 25 ml/min; KNAUER preparative Pump 1800, Berlin, Germany). Oil and methanol are pumped separately with two other HPLC pumps (methanol flow - KNAUER HPLC Pump K-501, Berlin, Germany; oil flow - Gilson 305 pump, Middleton, U.S.A). The three fluids are mixed in a static mixer (Kenics® 37-04-065, Chemineer, U.K.; 191 mm length and 3.4 mm inner diameter, with 27 mixings elements) before passing through the packed-bed enzymatic reactor (4.3 mL). Both the static mixer and the reactor are immersed in a water bath whose temperature is controlled to ± 1 ºC. The pressure in the reactor is controlled with a back pressure regulator (BPR) (Tescom Europe, model 26-1700, Selmsdorf, Germany) and measured with a digital pressure meter to ± 1 bar. The reaction mixture (monophasic) exiting the reactor is decompressed in the first separator (high pressure cyclone; Separex 4140/CY01AS1 Champigneulles, France). The pressure in this separator is controlled with a BPR and measured with an analytical pressure meter to ± 1 bar. Temperature is measured in-line with a Ni-CrNi thermocouple and controlled to ± 1 ºC. Two phases form: a liquid (bottom) phase where tri-, di- and mono-acylglycerols are recovered, with small amounts of FAME if separation conditions are not ideal, which is collected in a previously weighed trap immersed in an ice bath, and a gas (upper) phase consisting of CO_2, methanol, glycerol and FAME. The gaseous stream passes through a second (gravimetric) separator kept at ambient pressure and immersed in an ice bath, where FAME are recovered in the liquid phase, whereas

CO_2, methanol and glycerol remain in the gas phase. The flow of this gas stream is measured with a mass flow meter (RHEONIK RHM 007, Maisach, Germany) operating in the range 0.002 – 0.04 kg/min. Sampling with a chromatographic valve is provided at several points. Interconnecting lines use 1/8" O.D. tubing.

6.2.3. Analytical methods

The free fatty acid content of sunflower oil and WCO were determined by acid-base titration according to the standard method L 248/6 – 1991 (*Oficial Journal of European Communities No. 2568/91 L.248 September 5th*, 1991). FAME, triacylglycerols, di- and mono-acylglycerols, and by-products were measured by GC analysis performed with a Trace 2000 Series Unicam gas chromatograph with on-column injection, equipped with a 10 m x 0.32 mm i.d. column coated with a 0.10 μm thickness film of 5 %-phenyl and 95 %-dimethylpolysiloxane, from Zebron (Phenomenex, ZB-5HT Inferno column, California, U.S.A). The method used was the American Society for Testing and Materials (ASTM) standard method D6584 (*ASTM Standard D-6584-08*, 2008), with the operating conditions set as follows: the carrier-gas was helium at 0.1 ml/min for 1 min, followed by a 1.5 ml/min ramp up to 1.1 ml/min, holding until the end of the program; the oven temperature program was 50-80 ºC at 15 ºC/min, followed by a 7 ºC/min ramp up to 230 ºC, and a 30 ºC/min ramp up to 370 ºC, with a holding time of 10 min. A flame ionization detector was used, set at 380 ºC. Peak identification was carried out using known standards (FAME mixture). Heptadecane was used as internal standard. The data were processed with software *Excalibur*. No products were detected in assays carried out without enzyme. All the results presented are the average of at least two replicate experiments.

6.3. Results and discussion

6.3.1. Transesterification of virgin oil

Available phase equilibrium data relevant for binary and ternary systems (Bharath et al., 1992; Crampon et al., 1999; Güçlü-Üstündağ and Temelli, 2005; Hernandez et al., 2008; Inomata et al., 1989; Secuianu et al., 2008; Sovová et al., 1997) indicated that a single-phase system would prevail at the pressure and temperature conditions selected for the reaction experiments. Nonetheless, additional phase equilibria experiments were carried out and confirmed that at reaction conditions oil and methanol are totally soluble in $ScCO_2$.

An optimization of the reaction parameters was carried out for the continuous enzymatic transesterification of virgin oil with methanol, catalyzed by Lipozyme TL IM. At 20 MPa and 40 ºC, the solubility of sunflower oil in $ScCO_2$ was experimentally determined to be (4.5±0.2) g/kg_{CO2}. In the experimental apparatus used, the minimum oil flow rate possible is 40 μL/min,

leading to a minimum flow rate of liquid CO_2 of 10 mL/min. To allow for sufficiently high residence times, a reactor packed with 3 g of enzyme was used (an enzyme loading of 0.7 $g/mL_{reactor}$).

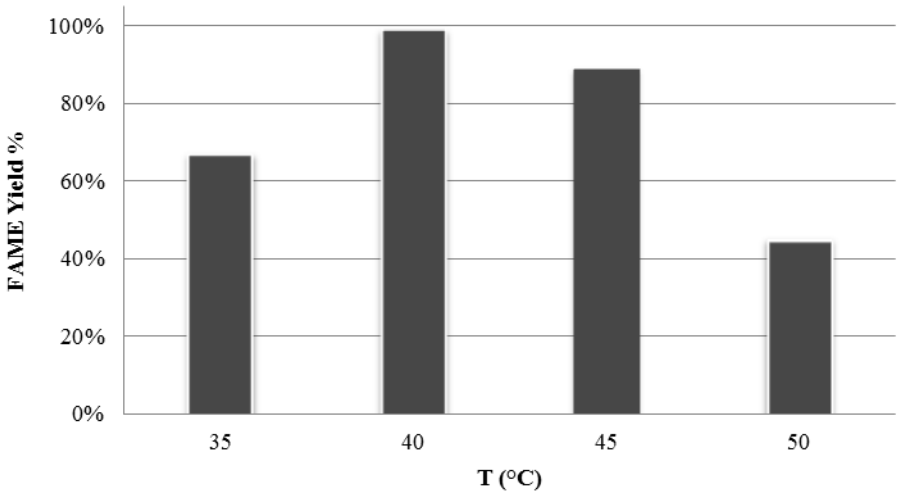


Figure 6.2: Effect of temperature on FAME yield. P = 20 MPa. Oil to methanol molar ratio = 1:29. Residence time = 20 s. Enzyme loading = 0.7 $g/mL_{reactor}$. Enzyme = Lipozyme TL IM.

Several experiments were conducted in a temperature range between 35 and 50 ºC. As seen in Figure 6.2, FAME yield increases up to 98.6 % as temperature increases to 40 ºC. The yield is still fairly high at 45 ºC, but then decreases. This behaviour is in general agreement with literature data that place the optimum temperature for the methanolysis of cooking oils between 40 and 60 ºC (Azocar et al., 2010; Y. Chen et al., 2009; Fjerbaek et al., 2009; Ognjanovic et al., 2009; Oliveira and Oliveira, 2001). Higher temperatures can cause enzyme deactivation.

In a continuous process, solvent flow rate and residence time are very important parameters in the design of the reactor. Knowing the optimum residence time, a balance between reactor volume and solvent flow rate can be made to optimize investment and production costs. Smaller residence times are more convenient for implementation at industrial scale, since they signify smaller reactor volumes and lower equipment costs. Experiments were performed with CO_2 flow rates in the range 7-15 mL/min (Figure 6.3), which corresponds to residence times between 14 – 28 s.

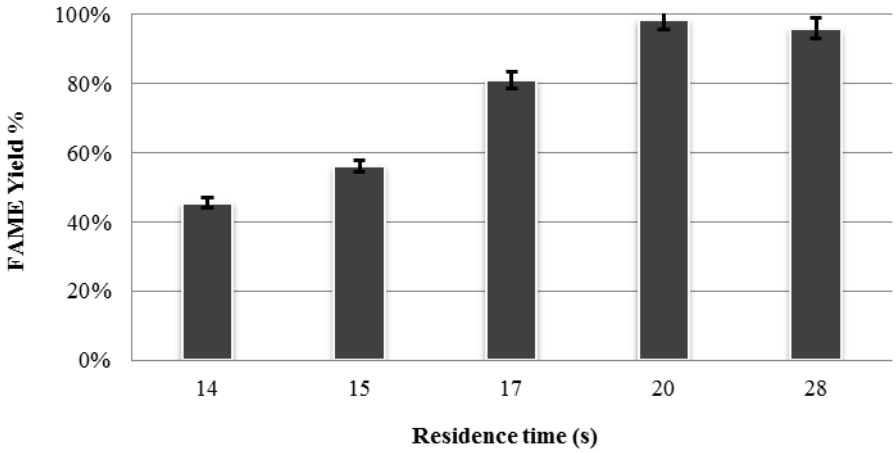


Figure 6.3: Effect of residence time on FAME yield. T = 40 ºC. P = 20 MPa. Oil to methanol molar ratio = 1:29. Enzyme loading = 0.7 g/mL$_{reactor}$. Enzyme = Lipozyme TL IM.

As expected, FAME yield increases as the residence time increases, and then levels out, as equilibrium is approached. The value obtained for the FAME yield for a residence time of 20 s is already so high that no additional gains are anticipated by further increasing residence time. Jackson and King (Jackson and King, 1996), who reached conversions above 98 % when working with Novozym 435, used residence times nearly 20 times larger than those studied in this work. Very high residence times are a drawback for an industrial application.

According to the transesterification reaction stoichiometry, 1 mol of oil reacts with 3 mol of methanol. However, in order to shift reaction equilibrium towards product formation, higher methanol to oil ratios must be used. Jackson and King (Jackson and King, 1996) report an optimum methanol to oil volumetric ratio of 4:5 in $ScCO_2$, which corresponds to a molar ratio of 1:25. The FAME yield obtained in the present work for a 1:24 molar ratio was 97 % (see Figure 6.4), in good agreement with the value obtained by Jackson and King (Jackson and King, 1996). We used concentrations of methanol between 60 and 180 mM, whereas Jackson and King used concentrations of methanol in the range 40-740 mM.

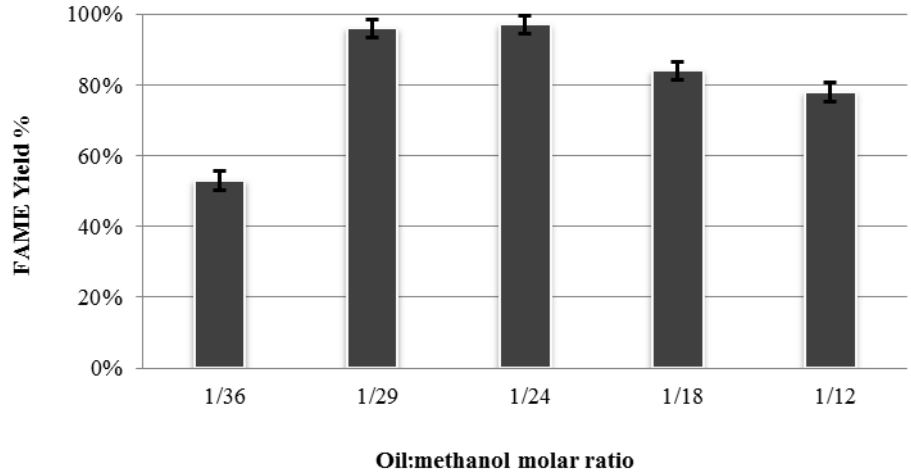


Figure 6.4: Effect of the oil to methanol molar ratio on FAME yield. T = 40 ºC. P = 20 MPa. Residence time = 28 s. Enzyme loading = 0.7 g/mL$_{reactor}$. Enzyme = Lipozyme TL IM.

In this work, experiments were performed for a maximum of 12 h, during which enzyme activity remained constant. This result was expected, as other authors have tested Lipozyme TL IM for long periods of time without any appreciable loss of activity (Hernández-Martín and Otero, 2008; Soumanou and Bornscheuer, 2009). Nevertheless, there were occasions when some malfunction forced us to shut down the system quickly, causing a sudden pressure drop that was seen to have a negative impact on the reaction conversion once the system was restarted. This may be due to the precipitation of oil on the enzyme, which is difficult to reverse by merely passing $ScCO_2$ through the enzyme bed.

6.3.2. Reaction product fractionation

The recovery of FAME from the reaction mixture requires a post-reaction fractionation stage, whereby temperature and pressure are varied in order to tune the properties of $ScCO_2$. This brings about changes in the solvation ability of CO_2 that enable the selective precipitation of dissolved species. A pressure range of 12-14 MPa and a temperature range of 40-60 ºC were tested in this work. At the reaction conditions that were found to maximize conversion in this work, the reaction mixture contains very small amounts of mono-, di- and triacylglycerols, which facilitates downstream separation. However, at these conditions, the rate of production of FAME is well below optimal. Higher rates of FAME production are achieved for higher flow rates, which bring along a decrease in the yield. To evaluate the role of $ScCO_2$ as facilitator of the separation and recovery of reaction products, we thus selected conditions yielding a moderate FAME yield of 55 %.

At constant pressure, the density of $ScCO_2$ decreases as temperature increases. Lower CO_2 density means lower solvation ability and higher selectivity. Therefore, as the temperature of the first separator increases at a constant pressure of 12 MPa, $ScCO_2$ becomes more selective for FAME, and the gas phase formed in the first separator becomes richer in FAME. This gas phase enters the second separator kept at ambient pressure and 0 ºC, where all the FAME are recovered as a liquid (Section 6.2.2). As Figure 6.5 shows, when the temperature of the first separator is 40ºC, 70 % of the liquid obtained at the end of the process are FAME. If that temperature is 60 ºC, the purity of the FAME obtained at the end of the process is 93 %. Taking into account that a given mass of triacylglycerols yields roughly the same mass of methyl esters, the gas stream exiting the reactor corresponds to a mass fraction of FAME of nearly 55 %. This shows that even when the first separator is kept at 40 ºC and 12 MPa, some degree of purification of FAME is achieved.

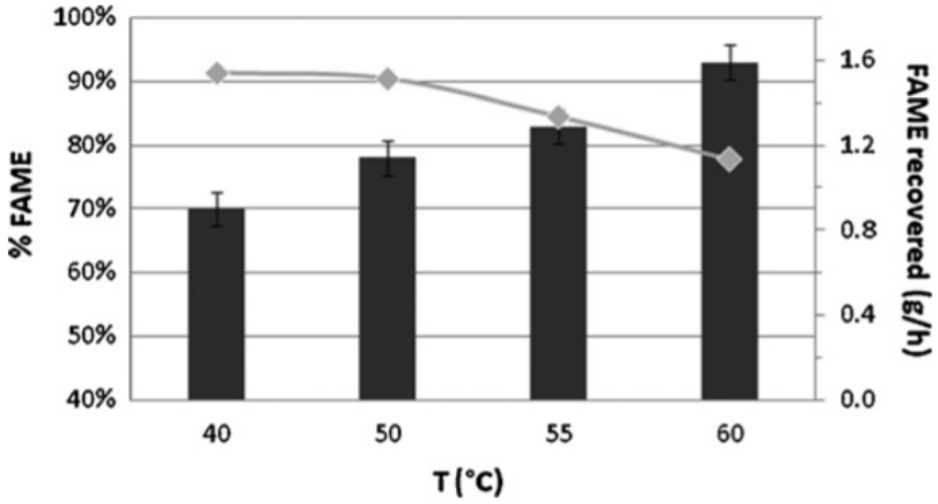


Figure 6.5: Effect of the temperature of operation of the first separator, kept at P = 12 MPa, on the recovery of FAME. The bars represent the mass fraction of FAME in the liquid phase recovered at the bottom of the second separator (Section 2.2). The grey line translates that quantity into the total amount of FAME obtained at the end of the process, per unit time. Reaction conversion was 55 %, which corresponds to a rate of production of FAME of 1.6 g/h.

Another aspect to take into account is the loading of the gas phase. Less dense $ScCO_2$ is more selective towards FAME, but since it has lower solvation ability, the amount of FAME it can dissolve is lower too. Thus, although when the first separator is kept at 12 MPa and 60 ºC FAME are obtained at 93 % purity, less FAME are obtained per unit time than when temperature is kept at 40 ºC. As indicated in the legend of Figure 6.5, the values given for FAME recovered compare with a rate of production of FAME in the reactor of 1.6 g/h. Thus at

60 ºC, only about 70 % of the total amount of FAME produced in the reactor are effectively recovered (ca. 1.1 g/h).

In Figure 6.6, we look at the effect of pressure on the separation and recovery of FAME. Now, temperature is kept constant at 60 ºC, and pressure is varied in the range 12-14 MPa. At constant temperature, increasing pressure brings about an increase in the density of $ScCO_2$. As referred earlier, this means lower selectivity in the separation, and recovery of FAME with a lower degree of purity (93 % at 12 MPa vs. 79 % at 14 MPa), although the amount of FAME recovered per unit time is higher (1.1 vs. 1.4 g/h).

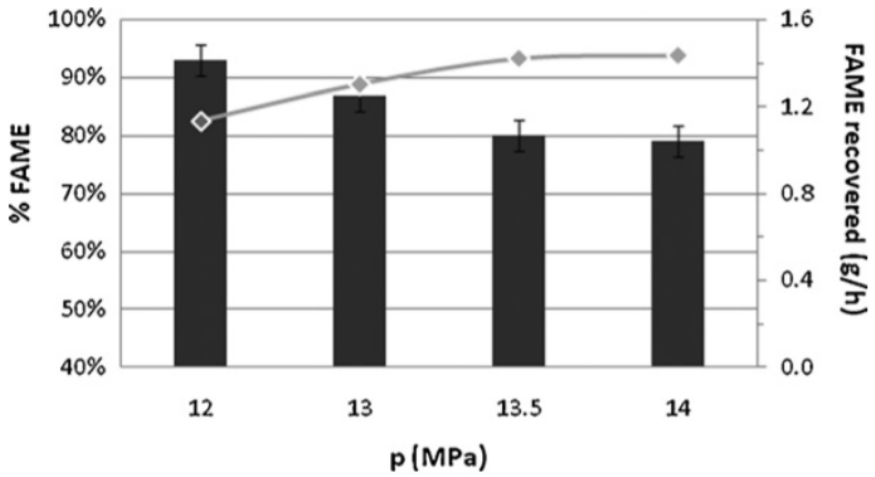


Figure 6.6: Effect of the pressure of operation of the first separator, kept at T = 60 ºC, on the recovery of FAME. Other conditions are as indicated in Figure 6.5.

For any given process, judicious choice has to be made regarding separation conditions. We have shown that in the case of less favourable reaction conditions or lower enzyme activity, the separation step has to be more selective, which means the use of pressures near 12 MPa and temperatures close to 60 ºC in the first separator. If only a small purification of the outlet stream of the reactor is needed, the first separator can be operated at higher pressures and lower temperatures, which lead to a higher amount of FAME recovered per unit time. According to the European Standard EN 14214, biodiesel must have > 96 % of FAME, a target that is easily met for yields slightly above 55 %. Any liquid collected in the first separator can be recycled back into the reactor.

The absence of methanol in the liquid phase of the first separator, as referred in Section 6.2.2. had been anticipated, based on available phase equilibrium data for relevant systems (Bharath et al., 1992; Crampon et al., 1999; Güçlü-Üstündağ and Temelli, 2005; Hernandez et al., 2008; Inomata et al., 1989; Secuianu et al., 2008; Sovová et al., 1997). The fact that methanol did not precipitate in the second separator must be due to the high flow rate of CO_2, as well as the comparatively much higher mass fraction of CO_2 in the gas stream. On the other hand, and as

mentioned in the Introduction, $ScCO_2$ can allow for efficient separation of glycerol from alkyl esters and acylglycerols due to pronounced differences in solubilities. By being the least soluble compound in $ScCO_2$ (Sovová et al., 1997), glycerol was expected to be recovered in the liquid phase of the first separator. Yet, it was not so, nor was glycerol detected in the liquid phase of the second separator. Evidence that glycerol did not stay in the enzyme bed was provided by recovering the enzyme from the reactor after experiments, suspending it in isopropanol, and analysing the supernatant by GC, to find no traces of glycerol. Instead, it was found that glycerol accumulated inside the BPR valve. This problem was overcome by heating up the BPR valve. But even then, glycerol did not precipitate in the two separators. This could be attributed to an entrainer effect of methanol, and the low amounts of glycerol produced in our lab scale apparatus.

6.3.3. Transesterification of WCO

WCO was also tested in the process. To evaluate the impact of the changes operated in WCO in the transesterification, we used the best reaction conditions determined for virgin oil and methanol (Figure 6.7).

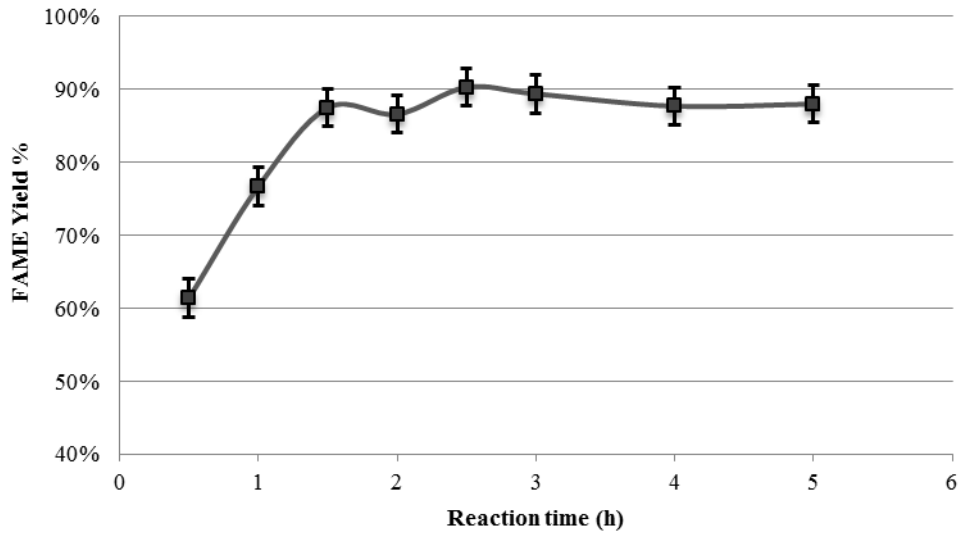


Figure 6.7: Reaction progress for the transesterification of WCO. T = 40 ºC. P = 20 MPa. Oil to methanol molar ratio = 1:24. Residence time = 20 s. Enzyme loading = 0.7 g/mL$_{reactor}$. Enzyme = Lipozyme TL IM.

Several physical and chemical changes occur in the cooking process. Many undesirable compounds are formed, whose toxicological effects are not fully known (Kulkarni and Dalai, 2006). These compounds are not easy to identify. Here we looked at humidity, acidity, and fatty acid profile.

Table 6.16: Comparison between virgin and waste cooking sunflower oil used in this work.

	Virgin oil	**Waste cooking oil**
Number of frying cycles	0	1-3
Water content (mg/g)	0.49 ± 0.02	0.71 ± 0.02
Acidity (as % w/w of oleic acid)	0.14 ± 0.07	0.66 ± 0.07

As seen in Table 6.1, cooking brings about a considerable increase in humidity, and also an increase in oil acidity. Sc-CO_2 is fairly dry, and it can easily accommodate the additional amount of water in WCO without any significant changes in water content (as seen earlier, the concentration of oil in the CO_2 stream is low). The enzyme is able to esterify the free fatty acids that are present in WCO. As for the fatty acid profile, cooking was found to not affect the oil content in oleic acid significantly, to bring about a slight decrease in linolenic acid, and an increase in palmitic acid. But overall, the degree of unsaturation of the oil did not decrease considerably. Still, FAME yield reached 89 %, which corresponds to a decrease of almost 10 % when compared to virgin oil.

Chen *et al.* (Y. Chen et al., 2009) report a reaction conversion of 94 % for WCO in *n*-hexane, using Novozym 435. Also in the batch methanolysis of WCO in *tert*-butanol using a combination of Novozym 435 and Lipozyme TL IM, Li *et al*. (Li et al., 2006) reached 95 % reaction conversion. We thus decided to include Novozym 435 in the process. Reaction conversion reached 99 % for a reactor configuration featuring the two enzymes, a value similar to the one obtained for virgin oil using Lipozyme TL IM alone. We note that, as shown in Figure 6.7, residence times above 20 s did not bring about significant changes in the reaction conversion. Thus, the increase in reaction conversion observed in Figure 6.8 is due to the addition of Novozym 435.

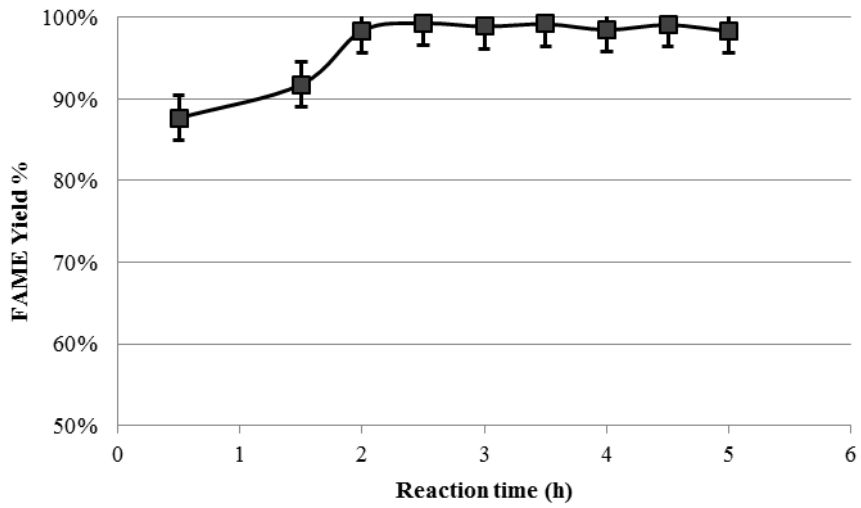


Figure 6.8: Reaction progress for the transesterification of WCO. T = 40 ºC. P = 20 MPa. Oil to methanol molar ratio = 1:24. Residence time = 30 s. Enzyme loading = 0.7 g/mL$_{reactor}$. Enzyme = Lipozyme TL IM and Novozym 435, at a 2:1 mass ratio.

6.4. Conclusions

We have looked at the production/recovery of biodiesel using a $ScCO_2$-based continuous, integrated process, comprising enzymatic synthesis via a methanolysis reaction followed by downstream separation. A balance must be sought between high reaction conversions, which may render unnecessary the fractionation of the reaction mixture leaving the enzymatic reactor but that do not lead to high rates of production of FAME, and intermediate reaction conversions that require the implementation of a downstream process for recovering the comparatively higher amounts of FAME produced per unit time. But even in the case of moderately favourable reaction conversions, we have seen that it is possible to obtain a product with >96 % of FAME using a $ScCO_2$-based process as described, whether the starting material is virgin or waste cooking sunflower oil.

7

General conclusions and future work

7.1. General conclusions

In this thesis, the potential of sub- and supercritical fluids combined with biotechnology was evaluated for the valorization of different lignocellulosic residues. HCW technology has proved to be a suitable technology to perform the valorization of different lignocellulosic residues with no significant commercial value. HCW has the potential to be used as a solvent for the extraction of added-value compounds directly available in the residues, such as, phenolic compounds and carbohydrates. It can also be used as a reactant for the hydrolysis of the lignocellulosic structure, converting structural polysaccharides, such as, cellulose and hemicellulose, into water soluble oligosaccharides and monosaccharides. The viability of using the soluble carbohydrates obtained in the liquors after HCW hydrolysis, as an alternative carbon source for microorganisms growth was also shown, representing the proof of concept regarding the main goal of this study.

RPS, containing a significant amount of cellulose and xylan, was used as the lignocellulosic material model to perform a detailed analysis of HCW hydrolysis process. In order to select the optimum operational mode for this process, experiments were performed using a batch and a semi-continuous flow reactor. Several operational parameters analysis as well as a detailed and exhaustive mass balances were performed for both operational modes. The mass balances performed enable an accurate analysis of lignin, hemicellulose and cellulose hydrolysis behavior through reaction time. The semi-continuous mode has proven to be the most fitted mode to perform HCW treatments. Higher amounts of soluble compounds including monosaccharides are obtained by performing HCW hydrolysis using a semi-continuous flow reactor at the same time that degradation reactions extensions are smaller corresponding to lower losses of hydrolyzed material. Additionally, since cellulose, hemicellulose and lignin are hydrolyzed at different temperatures, the semi-continuous mode can allow the fractionation of the main components by manipulating the operational temperature. For these reasons, further HCW treatments using grape pomace and apple pomace residues were studied using this operational mode.

GP represents an important residue rich in several added value products, which can be recovered by applying HCW treatment. HCW has proven to be an alternative environment friendly extraction solvent for phenolic compounds present in GP with extraction yields 10 times higher than when using conventional extraction methodologies. Moreover, HCW technology allowed the recovery of up to 60% of structural carbohydrates of GP, which were for the first time applied as single carbon source for the growth of non-Saccharomyces yeasts, exhibiting a good growth performance without performing any optimization. Therefore, the

hydrolysates obtained from HCW hydrolysis can be used for microorganisms growth as well as for further added value compounds accumulation such as carotenoids.

Considering the good performance observed for oleaginous yeast growth using the hydrolysates obtained from GP hydrolysis as single carbon source, another lignocellulosic residue was used to perform a more detailed study about not only growth performance but also for lipid accumulation. AP hydrolysis, a rich carbohydrate residue, was performed using HCW and the respective hydrolysates proved to be suitable for the growth of several oleaginous yeasts, also promoting lipid accumulation without any growth conditions optimization. Considering AP hydrolysis using HCW, a low hydrolysis temperature is needed in order to recover the maximum carbon structures solubilized, avoiding the presence of degradation compounds and turning the process economically more competitive. Therefore, a different strategy with three stages was proposed where (1) directly soluble monosaccharides are extracted using HCW at lower temperature followed by (2) a HCW hydrolysis performed at 130 ºC obtaining a liquor rich in oligosaccharides which can be further (3) hydrolyzed using enzymes.

The final goal of this process was to use the lipids accumulated by oleaginous yeasts to produced an alternative fuel. As final stage for the lignocellulosic residues valorization, a $ScCO_2$-based continuous, integrated process, comprising enzymatic methanolysis followed by downstream separation was proposed for lipids conversion into biodiesel. However, the amount of lipids obtained from AP hydrolysates assimilation was not enough to perform this study. Therefore, other alternative oil sources such as a virgin and waste cooking sunflower oils were used for proof of concept. With this integrated technology it was possible to obtain a product with >96 % of FAME proving the potential of using combined sub- and supercritical fluids and biotechnology to fully complete the valorization chain of a certain lignocellulosic residue.

However, the major drawback for HCW technology relies on the higher variability of the lignocellulosic residues. Several parameters can affect their main components resulting in different compositions and chemical structures, which will strongly affect HCW treatment efficiency. Therefore, each residue must be considered as unique and no standard conditions can be implemented. This results in the need for process optimization for each residue. Additionally, an important balance must be sough between the amount of soluble compounds recovered and the degradation reactions extension since the latter are unavoidable during HCW treatment. Depending on the final goal, it could be relevant to apply HCW as a pretreatment followed by other hydrolysis technologies, e.g., by using enzymes. For instance, several compounds derived from monosaccharides and oligosaccharides degradation reactions can have an inhibitory effect on microorganisms growth compromising process efficiency. Therefore, HCW process must be previously designed according with the final goal and the specificity of the residue in study.

7.2. Future work

In this work, three different lignocellulosic residues were considered to study the potential on using HCW treatment for their valorization, thus increasing their commercial value. Further work, related to this research area can be developed in the future, namely:

- Perform a scale-up of HCW treatment apparatus in order to obtained higher quantities of WSC'S which would allow not only to perform a more detailed analysis regarding the formation of degradation compounds but also to increase the amount of hydrolysates to be used as single carbon source for oleaginous yeasts growth and lipid accumulation.
- Evaluate the impact of other methodologies on HCW hydrolysis such a pH controlled treatment. Depending on the final goal, it can be considered an addition of a base to increase water pH and avoid acid catalyzed dehydration reactions of monosaccharides or, on the other hand, supercritical CO_2 which would acidify the medium promoting hydrolysis of more complex structures;
- Optimization of the residence time for HCW hydrolysis using the semi-continuous flow reactor in order to obtain a fractionation of the different oligosaccharides and monosaccharides along time minimizing their degradation;
- Optimization of the sample collecting system to recover the volatile degradation compounds which can be formed during HCW treatment;
- Development of a membrane process which would concentrate sugars recovered in the liquors avoiding the freeze-drying of the samples.
- Optimization of the mineral media, namely, the nitrogen source and carbon-to-nitrogen ratio as well as aeration in order to improve the cell growth for the selected oleaginous yeast strain;
- The evaluation of the potential of using the liquors for other possible applications mainly in the pharmaceutical and cosmetic industries regarding the phenolic compounds extraction from lignocellulosic residues using HCW;
- The application of this technology to several other lignocellulosic residues with significant impacts on the environment and actual society such as urban organic solid waste which is represent a major source of carbohydrates. It can also be applied in the valorization of other agro-industrial wastes with significant impact in Portugal such as residues derived from food industries such as white grape pomace and animal food processing (e.g. poultry and slaughterhouse wastes) or residues obtained in wood, cork and olive oil industries.

Further, scale-up process study and validation as well as technical-economic analysis are

required in order to evaluate the economic viability of the process.

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