NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

VALORIZATION OF GRAPE POMACE USING GREEN TECHNOLOGIES

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Master in Biotechnology

DOCTORATE IN CHEMICAL AND BIOCHEMICAL ENGINEERING

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Valorization of grape pomace using green technologies

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“Nothing is lost, nothing is created, everything is transformed” (Antoine Lavoisier).

ABSTRACT

Renewable and green resources need to be exploited to overcome the depletion and pollution problems caused by fossil resources, while fulfilling growing necessities for energy, food, and raw materials. The transition to a bio-based economy can be the solution for sustainable global development. The concept of circular bioeconomy aims to use biomass wastes and side streams efficiently in the sustainable production of high value products.

Grape pomace (GP) is the by-product generated by the wine industry. GP is composed mostly of lignocellulose, also containing proteins, lipids, sugars, and phenolic compounds. These high-value compounds can be recovered and applied in food, cosmetic or pharmaceutical industries.

The work presented in this thesis explores the complete valorization of GP using a cascade process, using subcritical water (SBW), for the fractionation of red wine grape pomace (RWGP) into differentiated extracts rich in phenolics, hemicellulose carbohydrates, cellulose carbohydrates, and the recovery of lignin. The phenolic-rich and hemicellulose-rich extracts were obtained using a semi-continuous reactor in a stepwise temperature program. Different temperatures were evaluated and the highest recovery yields were achieved at 100 and 190 ºC, respectively for phenolics and hemicellulose. The cellulose and lignin separation was achieved at 280 ºC, with the complete hydrolysis of cellulose and the loss of 49 % of lignin. At 250 ºC, 70 % was recovered and 90 % of the cellulose was hydrolyzed.

The obtained hemicellulose-rich extracts were studied as a source of prebiotics and in the formation of hemicellulose-based films. The extract exhibited prebiotic activity with the lactobacilli tested, having the best performance for *Lactobacillus paracasei*, with a prebiotic activity score of 0.4. The hemicellulose-based films were also successfully obtained, after a process of optimization and the addition of sorbitol and glycerol, as plasticizers.

Another goal of this thesis was to add value to the high amount of sugars readily available in white wine grape pomace. The yeast *Rhodotorula babjevae* was chosen to grow on this carbon source and produce different high-value products. *R. babjevae* was able to accumulate up to 23 % (w/w) of lipids with carotenoids (45 mg/100g_{oil}), and simultaneously secrete polyol esters of fatty acids (1.1 g/L in spent medium).

Keywords: grape pomace, subcritical water, lignocellulose fractionation, prebiotics, hemicellulose-based films, *Rhodotorula babjevae*.

RESUMO

Devido à escassez e poluição causada pelos recursos fósseis, a exploração de recursos mais sustentáveis, que permitam suprimir as necessidades energéticas, alimentar e de matérias-primas, tornou-se urgente. A transição para uma economia de base biológica pode ser uma solução para um desenvolvimento global sustentável. O conceito de bio economia circular visa a utilização de resíduos de biomassa e subprodutos na produção eficiente e sustentável de produtos de alto valor.

As massas vínicas (MV) são o subproduto gerado pela indústria vínica, sendo compostas, maioritariamente, por lignocelulose contendo também proteínas, lípidos, açúcares e compostos fenólicos. A recuperação destes compostos permite a sua aplicação na indústria alimentar, cosmética ou farmacêutica.

O trabalho apresentado nesta tese explora a valorização completa das MV usando um processo em cascata com água subcrítica, para o fracionamento das massas vínicas de vinho tinto (MVVT) em extratos diferenciados ricos em compostos fenólicos, hidratos de carbono de hemicelulose e de celulose, bem como na recuperação de lenhina. Os extratos foram obtidos usando um reator semi-contínuo, com um programa temperatura com diferentes patamares. Das diferentes temperaturas testadas, os melhores resultados foram obtidos a 100 e 190 ^{o}C, respetivamente para compostos fenólicos e hemicelulose. A separação da celulose e da lenhina foi conseguida a 280 ^{o}C, com a hidrólise completa da celulose e a perda de 49 % da lenhina. A 250 ^{o}C, recuperou-se 70 % de lenhina e 90 % da celulose foi hidrolisada.

Os extratos ricos em hemicelulose obtidos foram estudados como fonte de prebióticos e na produção de filmes à base de hemicelulose. Os extratos apresentaram atividade prebiótica com os lactobacilos testados, apresentando o melhor desempenho com *Lactobacillus paracasei*, com um *score* de atividade prebiótica de 0.4. Foi também possível obter filmes à

base de hemicelulose após um processo de otimização e da adição de sorbitol ou glicerol, como plasticizantes.

Outro objetivo da presente tese era acrescentar valor aos açúcares disponíveis nas massas vínicas de vinho branco. Para crescer nesta fonte de carbono e produzir diferentes produtos de elevado valor, escolheu-se a levedura *Rhodotorula babjevae.* A levedura foi capaz de acumular até 23 % (w/w) de lípidos com carotenoides (45 mg/100$g_{óleo}$) e, simultaneamente, produzir ésteres de poliol e ácidos gordos (1.1 g/L no meio de cultura).

Palavras chave: massas vínicas, água subcrítica, fracionamento da lignocelulose, prebióticos, filmes à base de hemicelulose, *Rhodotorula babjevae.*

LIST OF PUBLICATIONS

Papers published:

Bruno M. Pedras, Carla Gonçalves, Diogo R. Figueira, Pedro Simões, Paula Gonçalves, Alexandre Paiva, Susana Barreiros, Madalena Salema-Oom. "White wine grape pomace as a suitable carbon source for lipid and carotenoid production by fructophilic *Rhodorotula babjevae*" (2022). Journal of Applied Microbiology, 128, 138-144.

Bruno M. Pedras, Gabriela Regalin, Isabel Sá-Nogueira, Pedro Simões, Alexandre Paiva, Susana Barreiros. "Fractionation of red wine grape pomace by subcritical water extraction/hydrolysis" (2020). The Journal of Supercritical Fluids, 160, 104793.

Communications in conferences:

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Bruno Pedras, Isabel Sá-Nogueira, Pedro Simões, Susana Barreiros, Alexandre Paiva. "Fractionation of red wine grape pomace by subcritical water extraction/hydrolysis", 17th European Meeting on Supercritical Fluids, Ciudad Real, Spain, 8-11 April 2019 (oral communication).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS/ACRONYMS

1,6-HDO	1,6-hexanediol
5-HMF	5-hydroxymethylfurfural

A

acetyl-CoA	Acetyl coenzyme A
AFEX	Ammonia fiber explosion
ASE	Accelerated Solvent Extraction

B

BNC	Bacterial nanocellulose

C

CDW	Cell dry weight
CFU	Colony-forming units
CMC	Carboxymethyl cellulose
CNC	Cellulose nanocrystals
CNW	Cellulose nano whiskers

D

def-RWGP	Defatted RWGP
DES	Deep eutectic systems
DF	Dietary fiber
DPPH	2,2-diphenyl-1-picrylhydrazyl

E

EC_{50}	Half maximum effective concentration

F

FAs	Fatty acids
FDCA	2,5-furandicarboxylic acid
FOS	Fructooligosaccharides
FTIR-ATR	Fourier transform infrared-attenuated total reflection

G

GAE	Gallic acid equivalent
GC	Gas chromatography
GHG	Greenhouse gas
GOS	Galactooligosaccharides
GP	Grape pomace
GPC	Gel permeation chromatography

H

HE	Hemicellulose-rich extract

HE-A	Autoclaved hemicellulose-rich extract
HEC	Hydroxyethyl cellulose
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPC	Hydroxypropyl cellulose
HPLC	High pressure liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HVED	High Voltage Electrical Discharges

I

I_{preb}	Prebiotic index

K

K_w	Ionic product

L

LDL	Low-density lipoprotein

M

MAE	Microwave Assisted Extraction
MC	Methylcellulose
MFC	Micro fibrillated cellulose
Mha	Million hectares
MhL	Million hectoliters

MIC	Minimum Inhibition Concentration
Mn	Number-average molecular weight
Mp	Peak molecular weight
MRS	Man, Rogosa & Sharpe
Mt	Million tons
MUFA	Monounsaturated fatty acid
Mw	Weight-average molecular weight

N

NADPH	Nicotinamide adenine dinucleotide phosphate
NFC	Nano fibrillated cellulose
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy

O

OD	Optical density

P

PD	Polydispersity index
PEF	Pulse eletric fields
PEFA	Polyol esters of fatty acids
PHA	Polyhydroxyalkanoates
POH	Pulsed Ohmic Heating
PUFA	Polyunsaturated fatty acids

PYCC	Portuguese Yeast Culture Collection

R

rpm	Rotations per minute
RWGP	RWGP Red wine grape pomace

S

SBW	Subcritical water
SBWR	Hemicellulose-free residue
sc-CO_2	Supercritical carbon dioxide
SCO	Single-cell oil
SDGs	Sustainable Development Goals
SE-HPLC	Size Exclusion-High Performance Liquid Chromatography
SEM	scanning eletron microscopy
S:L	solid:liquid ratio
SLE	Solid-liquid extraction

T

T	Temperature
TAGs	Triglycerides/triacylglycerols
TGA	Thermogravimetric analysis
TOCSY	Total correlation spectroscopy
TPC	Total phenolic content
TSA	Tryptic Soy Agar

TSB	Tryptic Soy broth
U	
UN	United Nations
UV	Ultra-violet
W	
WWGP	White wine grape pomace
X	
XOS	Xylooligosaccharides
Y	
YPD	Yeast extract peptone dextrose

1

Introduction

1.1 Biorefinery and circular bioeconomy

The global population has been increasing at a rate of approximately 83 million people per year, and was expected to reach about 8.5 billion in 2030 and 9.7 billion in 2050 (Hassan et al., 2019; Kostas et al., 2021). The COVID-19 pandemic, which has led to an unprecedent health and economic crisis (Anghelache et al., 2022), may affect these projections, but still the trend is for an increase in greenhouse gas (GHG) emissions to the atmosphere (carbon dioxide, methane and nitrous oxide) due to human activity. The air pollution through GHG is already causing climate changes, affecting water quality and quantity, and human life, with extreme weather conditions such as high temperatures, heavy rains, hurricanes, and fires (Koruba et al., 2017).

The intensive exploitation and use of fossil resources is leading to a great increase of GHG emissions (Philippini et al., 2020). Fossil resources, such as oil, coal, and natural gas, have been used in the last century as the main source of energy, and have been essential for the economic and industrial development of many countries. Oil has also become the main resource for the production of many chemicals and materials. Plastics, for instance, became a material with a wide range of uses in daily life, but with the intensive use of these materials came high environmental impact, due to wrong waste disposal, creating pollution. The impact of plastics in the oceans, where plastic islands have appeared, is particularly severe. Moreover, plastics and microplastics can have a huge impact on animals and consequently on food and water quality (Conteratto et al., 2021).

In addition to environmental problems, there are issues regarding the availability of fossil fuel resources. In addition to not being bio-degradable, they are non-renewable, and their exhaustive use is leading to their depletion. One projection was that it would take less than 270 years to run out of petroleum on Earth, while it would take around 280 million years to replace it completely (Conteratto et al., 2021).

Renewable and green resources need to be exploited to overcome the depletion and pollution problems caused by fossil resources, while fulfilling growing necessities for energy, food, and raw materials. The main goal is to minimize the negative environmental impact and preserve

natural resources while maintaining global economic growth (Esteban et al., 2019; Kostas et al., 2021).

Alternative renewable resources are already being used to produce clean energy, such as wind, water, geothermal and photovoltaic power. However, this may not be enough for the increasing demand for energy as a result of growing population. Another option is the use of biomass, the most abundant renewable resource, for the production of marketable products, such as food, chemicals, materials and energy (Kostas et al., 2021).

The transition to a bio-based economy can be the solution for sustainable global development, having gained interest in the last years with multiple initiatives from the European Union, the United States, and South Africa (Kardung et al., 2021). The United Nations (UN) also launched, in 2015, the Sustainable Development Goals (SDGs) for 2030, which were adopted by all its members. The goals are focused on improving people's lives while preserving the planet for the future. The 17 SDGs include implementing measures to improve health and education and maintain economic growth, while paying special attention to the impact of those measures on climate changes, oceans, and forests (United Nations, 2016).

To achieve some of the SDGs, renewable bio-resources need to replace the conventional fossil resources, to ensure that sustainability, eco-efficiency, and global challenges can be met (Priefer et al., 2017). For that purpose, the concept of bioeconomy was created and can be defined by the production of materials, chemicals, and energy using plant or animal resources, instead of petroleum (Papadopoulou et al., 2021). It relies on agriculture, forestry, food processing, paper, chemical, and pharmaceutical industries, through the use of new technologies and biotechnological processes. New foods, feed, materials, chemicals and energy can be produced from these biogenic residues (Priefer et al., 2017). However, the transition to a bioeconomy will increase the demand for biomass, which can lead to intensive exploitation of land to obtain bio-resources. To avoid this, it is important to maintain sustainable biomass production and processing, which means maintaining the circularity of all processes and products (Gottinger et al., 2020). Circular economy can be defined as a concept of sustainability that gives priority to a more efficient use of resources, limiting and avoiding new resources as possible, also avoiding waste generation by recovering and recycling all the materials, in alternative to the linear economy framework of "take, make and dispose"

(Giampietro, 2019; Konietzko et al., 2020; Ubando et al., 2020). The term circular bioeconomy combines bioeconomy and circular economy and is an integrated concept that aims to use biomass wastes and side streams efficiently in the sustainable production of high value products (Leong et al., 2021; Mak et al., 2020).

The development of biorefineries is an important part of the transition to a bioeconomy. They are considered analogous to oil refineries, where in this case biomass is processed to obtain high value products. Initially, biorefineries were essentially focused on fuel production for transportation, bioethanol production from sugarcane in Brazil being one of the most well-known examples, as a reaction to the oil crisis in the 1970s. At the beginning of the 21st century, the production of biofuels was extended to biodiesel production from oilseeds processing. The 1st generation biofuels were based on minimal processing of plant biomass (Conteratto et al., 2021), which made these processes economically more attractive to compete with fossil energy. However, the use of land to obtain this biomass was a problem, since it could be used for food crops instead, which is a growing world need due to the increasing population. On the contrary, 2nd generation biofuels are obtained from residues from processed biomass, such as residues from biorefineries, forest biomass, agricultural crops, or other agro-industrial residues. Agro-industrial by-products are considered a "negative cost" waste from other industries. The transition to 2nd generation biofuels put an end to the controversy of "food vs. fuel" and turned the processes more interesting in terms of economy and sustainability (Hassan et al., 2019).

Biomass processing in biorefineries integrates different operations, such as pretreatment, saccharification, fermentation, or distillation. Green chemistry principles are always considered in the development of a sustainable process and in the design of biorefineries. The goal is to achieve a balance between efficiency and cost, environmental and health safety, ensuring economic viability and environmental sustainability (Islam et al., 2020). Economic viability while minimizing environmental impact is a great challenge when competing with traditional refineries. Hence, it is essential to integrate processes and combine multiple platforms to obtain different products in one facility, to use different feedstock wastes, to implement biorefineries close to other industries so that access to the residues generated is ensured with low transportation costs (Budzianowski and Postawa, 2016; Philippini et al., 2020).

1.2 Biomass

Biomass can be defined as all organic material of animal or vegetal origin that is biodegradable. It is essentially composed of carbon, hydrogen and oxygen, with smaller amounts of nitrogen, sulfur and chlorine, having solar energy stored in the chemical bonds (Koruba et al., 2017; Saxena et al., 2009; Tekin et al., 2014). Biomass can come from different sources, such as agricultural, forestry, municipal or industrial residues, and is the most abundant renewable resource on Earth. It has the potential to replace non-renewable fossil resources since it has a high oxygen content, and already contributes 10 to 14% to the world energy production (Lorenci Woiciechowski et al., 2020). Just in the food sector ca. 1.3 billion tons of residues are generated, food production having an efficiency of around 50% (Usmani et al., 2021). Agro-industrial residues are produced in large amounts around the world, during the processing of several types of crops. According to the UN report for circular economy and agribusiness development, 50% of the Earth's habitable surface is used for agriculture, and generates 25-30% of the global GHG emissions, similarly to the energy and industry sectors (US Environmental Protection Agency, 2022). Agriculture is additionally responsible for deforestation, land degradation and freshwater consumption (UNIDO, 2021). Due to their environmental impact, agro-industrial residues should receive appropriate treatment and be reused as much as possible for maximum benefit. However, most of them end up discarded in landfills or are incinerated, with only a small part being used for animal feed and composting. The various byproducts generated by the agro-industry have the potential to be used as raw materials in 2nd generation biorefineries to produce biobased products, having both economic and environmental benefits, since they can become a source of revenues and at the same time avoid the cost associated with their treatment and correct disposal (Batista Meneses et al., 2020; Philippini et al., 2020).

1.2.1 Lignocellulosic biomass

Agro-industrial residues/by-products are essentially composed of lignocellulosic material, the most abundant carbon source in the world. Lignocellulose is a complex, natural biopolymer, composed of three main structural components: cellulose, hemicellulose, and lignin (**Figure 1.1**). The proportions of these three components vary, depending on the source. Lignocellulose

is mostly present in the plant cell wall. As a result of the interaction between its constituents, lignocellulose is a very resistant, stable, and compact polymer, making it very challenging to deconstruct and isolate its components (Lorenci Woiciechowski et al., 2020).

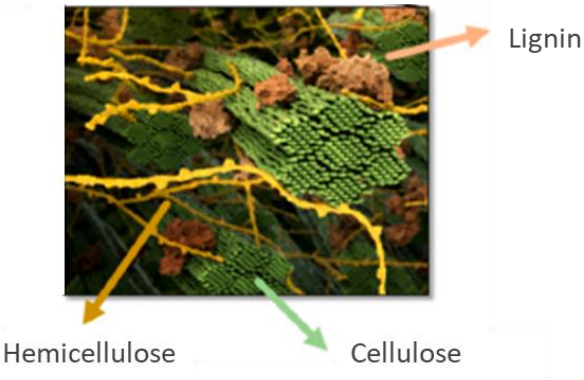

Figure 1.1 - Schematic representation of a lignocellulose matrix (adapted from Ganewatta et al. (2019)).

1.2.1.1 Cellulose

Cellulose is the most abundant natural polymer in the world, making its applications very attractive. Cellulose is biodegradable, biocompatible, non-toxic, has good mechanical properties and low solubility in water, and can also be easily modified (Batista Meneses et al., 2020). Structurally, cellulose is a linear homopolymer with high molecular weight (ca. 500,000 g/mol). It is composed of more than 10,000 units of glucose linked through (β-1-4)-glycosidic bonds, with the molecular formula of $(C_6H_{10}O_5)_n$, represented in **Figure 1.2** . Cellobiose is a dimer of two glucose molecules and is the main unit of cellulose. Cellulose is arranged in multiple layers allowing hydrogen bond and van der Walls interactions between chains, creating microfibrils formed by microcrystals, which make cellulose a very stable structure (Nagarajan et al., 2017; Tekin et al., 2014).

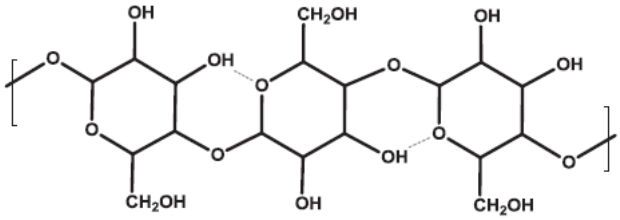

Figure 1.2 - Structure of cellulose (adapted from Tekin et al., (2014)).

1.2.1.2 Hemicellulose

Hemicellulose is a heteropolymer composed of different sugar monomers, namely pentoses (C5) and hexoses (C6). The pentoses comprise xylose and arabinose, and the hexoses comprise mannose, galactose, and glucose (**Figure 1.3**). The proportions of these monomers differ, depending on the plant type and species. Compared to cellulose, hemicellulose has an amorphous structure and is a random, branched polymer, most commonly with a xylan (polymer of xylose units) or galactomannan (polymer of galactose and mannose units) backbone, and side chains composed of the different C5 and C6 sugars. Hemicellulose also has a shorter chain length, with 100-200 sugar units. Hemicellulose makes hydrogen bonds with cellulose and covalent bonds with lignin, which helps to maintain the stability of the lignocellulosic structure. Due to its lower degree of polymerization and branched structure, it is more susceptible to hydrolysis (Tekin et al., 2014).

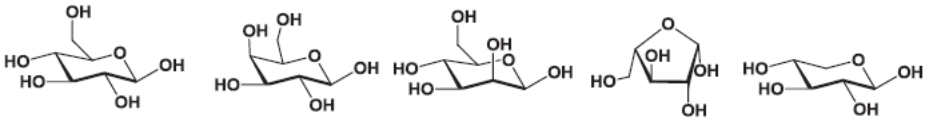

Figure 1.3 - Sugar units of hemicellulose. From left to right: glucose, galactose, mannose, arabinose and xylose (adapted from Tekin et al., (2014)).

1.2.1.3 Lignin

Lignin is the third main component of lignocellulose in plant cell walls and is a major source of aromatic compounds. It is essentially composed of phenols, instead of sugar units. Lignin can be classified as guaiacyl (G), syringyl (S), or p-hydroxyphenyl (H), depending on the phenols present being mostly coniferyl, synapil, or p-coumaryl alcohols, respectively (**Figure 1.4**). Depending on the origin of biomass, lignin can have a wide range of molecular weights between 1,000 and 20,000 g/mol. Guaiacyl is more predominant in softwoods, hardwoods

contain guaiacyl and syringyl lignin, and grasses have all three monomers (Batista Meneses et al., 2020; Lorenci Woiciechowski et al., 2020). Lignin is a hydrophobic polymer responsible for providing stiffness and hardness to plants. It covers cellulose and hemicellulose, controlling permeability and improving cell mechanical properties, which protects them from chemical and microbial attack (Lorenci Woiciechowski et al., 2020). Lignin is also used by plants to store energy, being responsible for the high heating value of biomass (Tekin et al., 2014).

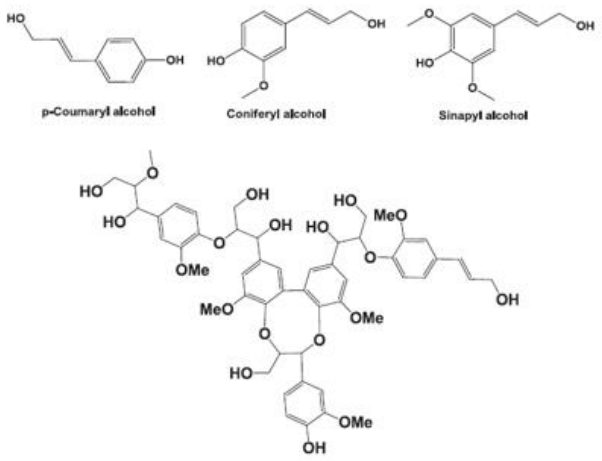

Figure 1.4 - Basic lignin monomers and the structure of a sample fraction (adapted from Tekin et al. (2014)).

1.2.1.4 Other components

In addition to cellulose, hemicellulose and lignin, agro-industrial residues also have non-structural components, which are present in lower amounts. These include simple sugars, proteins, lipids, minerals and other micronutrients, such as phenolic compounds.

1.2.2 Grape pomace

One example of agro-industrial residues/by-products is grape pomace generated by the wine industry, which is the subject of this thesis.

Wine production is one of the most important agro-industries in the world. Consequently, grapes, mainly *Vitis vinifera,* are one of the most abundant crops in the world, with about 7.4 million hectares (Mha) of land used for cultivation, and a grape production of 77.8 million tons (Mt) in 2018. Of these, 57% were wine grapes, 36% were table grapes, and 7% were dried

grapes. The major grape producer in the world was China, with 11.7 Mt, followed by Italy (8.6 Mt), the United States (6.9 Mt), Spain (6.9 Mt), and France (6.2 Mt). However, China mostly produces table grapes, while the other counties channel most of their production towards winemaking. In 2018, 292 million hectoliters (MhL) of wine were produced, with Portugal being the 11th world producer with 6.1 MhL, and 10th wine exporter with 3 MhL, values that represent an increase of 5% between 2014 and 2018 (OIV, 2021, 2019).

The winemaking process starts with harvesting. Grape clusters are cut from the vine and transferred to the winery, where destemming and crushing occurs. In the white winemaking process, crushed berries are pressed and separated from the juice, which proceeds to fermentation. On the other hand, in red winemaking the crushed berries are submitted to the fermentation step together with the juice, which allows the extraction of pigments, such as anthocyanins, from the skins to the juice, giving wine a strong red color. After fermentation, the juice is separated from the solids by pressing the must (Amerine, 2021). Finally, the wine is settled, clarified, filtered, and bottled. Red wines can additionally be matured for up to 3 years in oak barrels, which improves their flavor, acidity, alcohol content, and provides additional clarification and stabilization (Beres et al., 2017). The wine can also continue aging in the bottle, for 2 to 20 years. Nevertheless, most of the table wines do not need aging and are consumed before being two years old.

To produce special wines, such as sparkling and fortified wines, additional steps are included in the process. For sparkling wines, the two techniques used are direct carbonation of the wine during bottling, or second fermentation in the bottle, where enough sugar is added and the final alcohol content increases by about 1%. Fortified wines are produced by adding alcohol during or after fermentation, which will increase the alcohol content and avoid the complete fermentation of sugars. The excessive alcohol is then distilled to reach an alcohol content between 17 to 21%. The wine sweetness depends on the fortification timing. Very sweet wines, such as muscatels, are fortified earlier, while Madeira wines are fortified at the end of the fermentation, being subjected to a baking process (four months at 58-65 ^{o}C), ending with a more caramelized flavor (Amerine, 2021).

The residues/by-products generated during the winemaking process are plant remains, grape pomace after pressing (white wine) and grape pomace after pressing and fermentation (red

wine), and lees obtained during sedimentation, consisting essentially of dead yeast. Grape pomace (GP) is the major by-product generated, representing 15-20% of the total grape input (**Figure 1.4**). It is composed mainly of stems, skins, residual pulp, and seeds. GP has been undervalued, traditional uses being the production of spirit beverages, its use in animal feed, or for compost, which together account for only 3% of the total GP. Most of GP is discarded in landfills or burnt, which can be harmful to the environment, since the compounds present are resistant to biological degradation, causing surface and ground water contamination. Other associated problems are the attraction of flies/pests that can spread diseases (Bordiga et al., 2019). To avoid pollution, companies are forced to treat their residues, which represents an additional cost. In Spain, wine companies are already obliged to recycle, valorize, or dispose adequately of their residues. Most choose to pay for disposal. However, big penalties are charged if disposal is not done properly, following specific rules. These policies are being implemented to promote good waste management and sustainability of the winemaking process, by adding value to the winery residues/by-products and avoiding pollution (Beres et al., 2017).

Figure 1.5 - Dried samples of red and white grape pomace.

1.2.2.1 Grape pomace composition

GP is composed of grape pulp (ca. 60%) and grape skins (ca. 40%). The main components of GP are dietary fiber, proteins, lipids, minerals, and bioactive compounds, such as phenolic compounds.

Dietary fiber (DF) is all edible parts of plants that are resistant to human digestion and absorption in the small intestine and are only completely or partially fermented in the large intestine. DF consumption is associated with a lower risk of cardiovascular disease, cancer and

diabetes (Deng et al., 2011). GP dietary fiber is essentially composed of the structural components cellulose, hemicellulose and lignin. It comprises 43 to 75% of the total GP, being more abundant in seeds than skins, and in red wine grape pomace (RWGP) when compared with white wine grape pomace (WWGP). This difference is a result of the winemaking process, since in white winemaking, GP is removed before fermentation (Deng et al., 2011). This leads to the presence of a large amount of reducing sugars (ca. 40% of WWGP), mainly glucose and fructose, and a corresponding lower amount of structural components. RWGP, on the other hand, has only a low percentage of reducing sugars. The other monosaccharides found in GP are xylose, mannose, galactose, arabinose and rhamnose (Bordiga et al., 2019).

GP proteins can vary between 6 to 15%, depending on the variety and culture conditions, and have similar amounts in skins and seeds. Although GP proteins are not extensively studied, the amino acid profile shows similarities with those found in cereals, with high levels of glutamic and aspartic acid and low levels of sulfur amino acids, such as tryptophan and cysteine.

The minerals present in GP have a high variability depending on environmental and cultivation conditions, as well as the winemaking process. Some of the minerals found are potassium, phosphorus, sulfur and magnesium, tartrate being the most abundant potassium salt (Bordiga et al., 2019).

Lipids are mostly present in seeds, with grape seed oil being widely used in cosmetic applications. Grape seeds have 8 to 15% of lipid content, with a fatty acid profile mostly composed of unsaturated fatty acids such as oleic and linoleic acids, which together account for more than 89% of the total oil content. Linoleic acid is the most abundant (60-78%), followed by oleic acid (10-24%). Palmitic and stearic acids are the most abundant saturated fatty acids with a variation of 7-9% and 3-6%, respectively. Additionally, β-sitosterol and α-tocopherol are the main sterol and tocopherol present, respectively (Beres et al., 2017). β-carotene and other sterols, such as stigmasterol and sitostanol, are also present in grape seed oil, having mainly antioxidant and anti-arteriosclerotic activities, respectively. The main application of grape seed oil is in cosmetic formulations for damaged and stressed skin tissues, due to its regenerative and restructuring qualities associated with high antioxidant and sterol contents, making it ideal for skin care. If used as a food additive, it can also be hepatoprotective

and neuroprotective, and help in the reduction of liver cholesterol (Beres et al., 2017; Yu and Ahmedna, 2013).

Phenolic compounds (phenolics) are one of the largest and most important groups of natural products produced by plants under stress as secondary metabolites, to protect against UV radiation, pathogens, and other environmental threats. Phenolics are well known for their antioxidant, antimicrobial, anti-inflammatory, antithrombotic, antiallergenic, cardioprotective and vasodilatory activities, having been associated with health benefits promoted by vegetable and fruit consumption (Barba et al., 2016; Beres et al., 2017). They are also responsible for astringency, color and odor of plant foods (Bertelli et al., 2021).

Grape, apple, blueberry, and cranberry are considered much richer in phenolics than any other fruits and plants. In GP, most phenolics are present in seeds (60-70%), followed by skins (28-35%) and pulp (less than 10%). These compounds are extracted during the winemaking process, providing health benefits associated with a moderate wine consumption. However, only 30-40% of the total phenolic compounds are extracted during vinification, leaving behind a residue extremely rich in bioactive compounds that can be further extracted, adding great value to the wine by-product (Beres et al., 2017; Yu and Ahmedna, 2013).

Chemically, phenolics are molecules with one or more phenyl rings and one or more hydroxyl groups, and according to their chemical structure, phenolics are classified in classes and sub-classes. The main classes are phenolic acids, flavonoids, tannins, lignans, and stilbenes (Yu and Ahmedna, 2013).

Phenolic acids are the second most important group of phytochemicals and are simple compounds composed of an aromatic ring with a carboxylic acid functional group that gives them an acidic character. They are divided in two sub-classes: hydroxycinnamic acids, and hydroxybenzoic acids. The most common hydroxycinnamic acids found in GP are ferulic, p-coumaric, and caffeic acid, while the hydroxybenzoic acids are gallic, protocatechuic, and syringic acids (Beres et al., 2017; Fontana et al., 2013; Haminiuk et al., 2012).

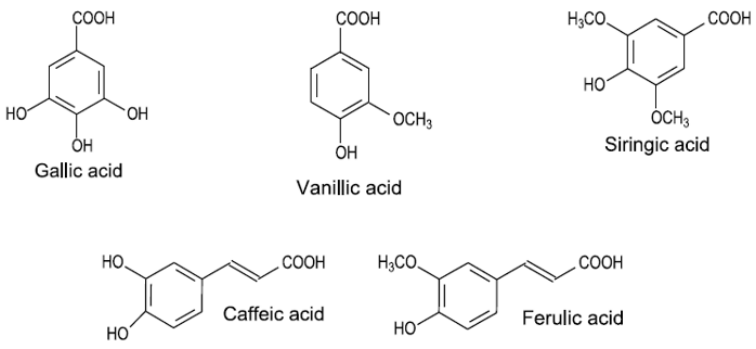

Figure 1.6 – Examples of phenolic acids found in GP (adapted from Bodoira and Maestri (2020)).

The largest and most important group of phenolic compounds are flavonoids, with more than 5,000 structures identified and accounting for more than two thirds of dietary phenols. Flavonoids are responsible for yellow, red, and blue colors in fruits and can be divided into seven sub-classes: flavones, flavanones, flavonols, isoflavones, anthocyanins, flavanols, and chalcones (Balasundram et al., 2006). Flavonols are the most abundant flavonoids in nature, being present in grape skin and seed. White grapes are richer in gallocatechin, procyanidins, catechin and epigallocatechin. Quercetin, kaempferol and myricetin are mostly found in glycosylated forms (Beres et al., 2017; Makris et al., 2006).

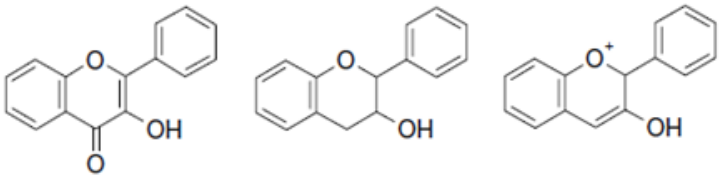

Figure 1.7 - Generic structure of the main classes of flavonoids, from left to right: flavonol, flavanol and anthocyanidin (adapted from Balasundram et al. (2006)).

Tannins are a group of high molecular weight phenolics and can be divided into condensed, and hydrolysable tannins. The former consists of polymers of flavonols, while the latter are esters of gallic acid.

Lignans are non-flavonoid compounds derived from cinnamic acids and comprise a large variety of different compounds, representing one of the major classes of phytoestrogens. However, they are not abundant in fruits (Haminiuk et al., 2012; Moss et al., 2000).

Stilbenes are not abundant in plants and fruits and resveratrol and its derivates are the only compounds of this class in GP, being present in both free and glycosylated forms (Fontana et al., 2013; Soto et al., 2015).

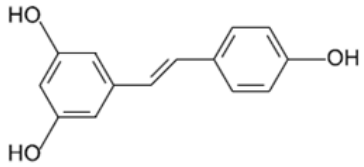

Figure 1.8 - Trans-resveratrol, the main stilbene found in GP (adapted from Bodoira and Maestri (2020)).

The structure of a phenolic compound plays an important role in its antioxidant activity, which is related to the capacity of scavenging free radicals and donating hydrogen atoms or electrons. In phenolic acids, the simpler molecules, the antioxidant activity is directly correlated with the number of hydroxyl groups and their positions in relation to the carboxyl group (Balasundram et al., 2006). Flavonoids usually have higher antioxidant activity than phenolic acids, stilbenes and lignans due to their structure and the number of hydroxyl groups present.

GP, considered a great source of phenolic compounds, is abundant in anthocyanins, hydroxybenzoic and hydroxycinnamic acids, flavonols, flavanols and stilbenes, present mainly in skins and seeds (Bordiga et al., 2019). However, within the same grape variety, significant differences in the phenolic composition can be found. Their content in GP is also dependent on the vinification method since longer contact times produce a lower content in pomace. As in other plants, grape pomace has a large amount of non-extractable phenolic compounds that cannot be recovered under mild conditions. These phenols are essentially highly polymerized condensed tannins and phenolics complexed with fiber, proteins, or membrane polysaccharides, and can only be extracted after hydrolysis of GP (Yu and Ahmedna, 2013).

1.3 Valorization of agro-industrial by-products

The valorization of GP and other agro-industrial by-products can be done using the raw material as such. A major application is in the field of energy, where combustion of lignocellulosic biomass is probably the oldest method to produce energy. Even with the emissions of CO_2 generated, it can be considered green because it is carbon neutral.

Combustion generates the same amount of carbon dioxide that was fixed by the plant biomass. However, with the current need to reduce GHG emissions, it is not the optimal option to exploit (Chrysikou et al., 2018).

In composting, lignocellulosic biomass is decomposed aerobically into added-value products, such as humic substances, by the action of endogenous microbes and enzymes. The low efficiency of the process is the main barrier for its industrial implementation (Usmani et al., 2021; Wu et al., 2022).

In anaerobic digestion, microorganisms produce biogas from the degradation of organic substrates present in lignocellulosic materials. It is a complex biological process with four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Different bacteria are involved in the different stages of the process, to achieve the final product, biomethane (Faisal et al., 2021).

Lignocellulosic material can also be used to produce biohydrogen. The direct production of hydrogen using specific bacteria, such as *Clostridium thermocellum*, can be an attractive alternative, since it is cost-effective, commercially feasible and biohydrogen combustion is pollution free, since only water vapor is produced (Cheng et al., 2011; Singh et al., 2021).

Pyrolysis is a thermo-chemical process used to decompose biomass at temperatures between 500 and 800 ^{o}C, in the absence of oxygen. From this process are obtained char, gas, and vapors/aerosols that are condensed to a liquid - bio-oil. Bio-oil is a complex mixture of low molecular weight molecules (acids, esters, aldehydes, ketones, phenols) and can have different applications in the energy sector. Bio-oil has been used in boilers, furnaces, and electricity generators (Hassan et al., 2019; Thangalazhy-Gopakumar et al., 2010).

Other applications of biomass require a pretreatment aiming at the fractionation of the lignocellulose into its main structural components and depolymerization (**Figure 1.9**).

1.3.1 Lignocellulose pretreatment

As mentioned earlier, lignocellulose is composed of cellulose, containing both crystalline and amorphous regions, combined with amorphous hemicellulose chains, enwrapped in lignin. Due

to the characteristics of these polymers, lignocellulose depolymerization and recovery of the isolated main components is very difficult.

Cellulose and hemicellulose are polysaccharides. Enzymatic hydrolysis is the most common method to break down such polymers. However, due to biomass recalcitrance, enzymatic hydrolysis it is not effective without biomass pretreatment. Pretreatments can be the most expensive step in lignocellulosic materials conversion and can be classified as physical, chemical, physicochemical, or biological. Physical and chemical processes are usually more efficient, but require more equipment and generate more pollution, while biological processes use less energy and are less pollutant, but have lower efficiency and are more expensive (Chen et al., 2017).

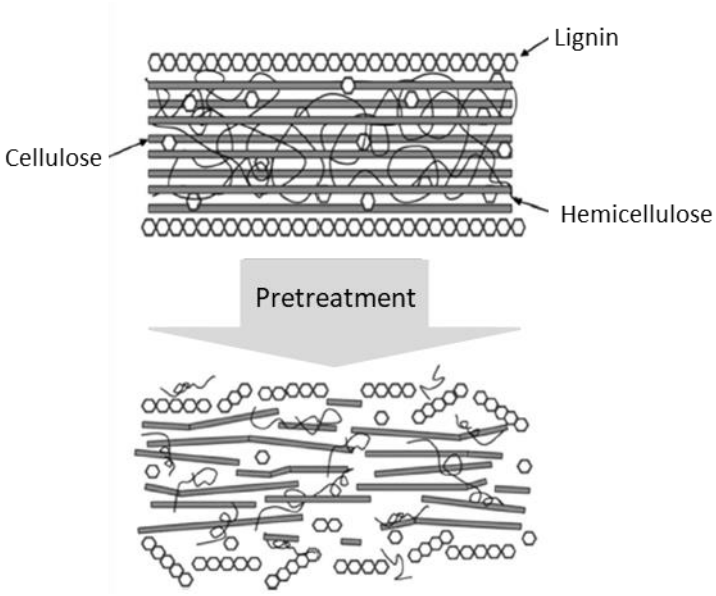

Figure 1.9 – Schematic representation of lignocellulose pretreatment (adapted from Haghighi Mood et al. (2013)).

1.3.1.1 Chemical pretreatments

Chemical pretreatments include mainly acid and alkaline processes. In acid pretreatments, concentrated or dilute inorganic acid (sulfuric, hydrochloric, phosphoric and nitric acids) and organic acid (acetic, citric and oxalic acids) solutions are applied. Chemical pretreatment is considered a very effective technique, with some drawbacks, such as corrosion, toxicity, and high environmental hazard. Sulfuric and hydrochloric acids are most efficient in these processes. Although sulfuric acid is more toxic, it is preferred over HCl whose use requires corrosion-resistant materials. Dilute acid solutions (below 10%) are preferred for hydrolysis of

biomass since the main goal of an acid pretreatment is to solubilize the hemicelluloses, and part of lignin, to make the cellulose more susceptible to enzymatic attack, by decreasing its crystallinity. These processes appear to be more favorable for industrial applications, because they generate a low amount of degradation products, hemicellulose is completely hydrolyzed into its monomers, and the acid does not need to be recycled (Chen et al., 2017; Lorenci Woiciechowski et al., 2020; Singh et al., 2014). However, concentrated acid solutions can also be applied with the goal of high sugar recovery yields, aiming at hydrolyzing both hemicellulose and cellulose completely into their monomeric sugars. This approach has some drawbacks when the liquor obtained is used as carbon source for a microbial application, since sugars can be degraded and converted into toxic levels of compounds such as acetic acid, furfural and 5-hydroxymethylfurfural (5-HMF), requiring removal before application (Lorenci Woiciechowski et al., 2020).

Alkaline pretreatments are very effective in the delignification of biomass, maintaining the cellulose structure intact, being the traditional method used in pulp and paper processing. They use less aggressive reagents when compared to acid pretreatments. Alkaline pretreatments allow the dissolution of lignin and part of hemicellulose, by breaking chemical and hydrogen bonds between the components and destroying the lignin structure. The degree of polymerization decreases, modifying the surface area, porosity and crystallinity (Batista Meneses et al., 2020; Lorenci Woiciechowski et al., 2020). However, as in acid pretreatments, alkaline hydrolysis can lead to the degradation of sugars, even though in smaller amounts, being more efficient in biomass with low amounts of lignin. Sodium hydroxide, potassium hydroxide, and calcium hydroxide are the main alkalis used. Calcium hydroxide is the most hazardous to work with but has a lower cost in comparison to sodium and potassium hydroxide (Chen et al., 2017).

Other chemical processes use ionic liquids and organosolv as pretreatment. Ionic liquids, mainly imidazole ion salts, are very effective in cellulose dissolution, by breaking hydrogen bonds in the lignocellulosic structure, allowing the extraction with water, ethanol or acetone, but with high processing costs (Batista Meneses et al., 2020). Organosolv pretreatment is more focused on lignin removal. It uses organic solvents such as ethanol, methanol, or glycerol, with or without water, and an acid catalyst such as hydrochloric, or sulfuric acid, or an organic acid.

Conducted at high temperatures (100-250 ºC), organosolv promotes hemicellulose hydrolysis and lignin extraction and solubilization in the organic solvent. This method also produces some sugar degradation and specific equipment is needed due to the use of a volatile organic liquid that has an inherent risk of fire and explosion (Agbor et al., 2011). To avoid the use of hazardous chemicals in organosolv, high pressure carbon dioxide can be used as acidifying agent instead of the strong acids usually used (Gurgel et al., 2016).

1.3.1.2 Physical pretreatments

Physical pretreatments consist of mechanical crushing, ultrasound, and microwave treatment. These methods have a lower environmental impact and are relatively simple but have high energy requirements.

Milling processes are used to reduce the particle size and disrupt the lignocellulosic structure. They are normally used before other methods and can generate particles between 0.2 and 2 mm, which increases the surface area, and decreases processing time and water consumption, thereby improving hydrolysis.

Sonication consists in using ultrasound waves to create microcavities and bubbles that produce shock waves when collapsing, causing the reduction of particle size due to erosion (Batista Meneses et al., 2020). This process can decompose lignin, hence improving accessibility to cellulose, and it can also depolymerize hemicellulose chains.

Extrusion consists in molding biomass particles, by forcing them to pass through a mold with a desired cross-section. This reduces particle size, increases the specific surface area and changes crystallinity (Batista Meneses et al., 2020).

Microwaves can be an energy-efficient method to heat in a short time and can be combined with other methods to change the physicochemical properties of the biomass. The major drawback are the high equipment cost (Chen et al., 2017). However, microwave radiation can avoid temperature gradients and overcome solid concentration limits in stirred reactors, since heating is due to the penetration of microwaves in the liquid by creating an electromagnetic field (Singh et al., 2014).

1.3.1.3 Biological pretreatments

Lignin degradation can be accomplished using bacterial or fungal species. The main advantages of these processes are their low cost, no use of chemicals, and absence of pollution. The major disadvantage is the long incubation periods needed, which is not industrially attractive, since the efficiency is always low, due to lignocellulose recalcitrance. As mentioned earlier, enzymatic hydrolysis, also a biological approach, is much more efficient after a chemical or physical pretreatment, as a means to complete hemicellulose and cellulose hydrolysis and obtain the monomeric sugars (Batista Meneses et al., 2020; Chen et al., 2017).

1.3.1.4 Physicochemical pretreatments

Physicochemical processes include steam explosion and hydrothermal treatment. Steam explosion consists in treating biomass with hot steam (180-240 ^{o}C) under pressure (10-35 bar) in a reactor. The pretreatment has two stages. The first autohydrolysis occurs when the water vapor condenses and penetrates the biomass, starting the hydrolysis of hemicellulose due to the formation of acetic acid that catalyzes the reaction. The second stage consists in the fast depressurization of the reactor, which causes the explosion of biomass and consequently reduction of particle size. This process allows to achieve a higher depolymerization of lignocellulose by hydrolyzing hemicellulose and decomposing lignin. It is a process with low environmental impact, but it still requires optimization of the reactor and operation conditions, since some toxic compounds can be generated from sugar degradation. Steam explosion can also be combined with alkali pretreatment, in ammonia fiber explosion (AFEX), involving the use of anhydrous ammonia at high temperatures and pressures. The major advantage of this process is that it can decrease the production of degradation products that can act as fermentation inhibitors when the liquor produced is intended for a microbial application. However, ammonia needs to be recycled since it is not environmentally friendly and is expensive to obtain (Arya and Rao, 2007; Batista Meneses et al., 2020; Chen et al., 2017; Singh et al., 2014). Supercritical CO_2 explosion can be used, similarly to the steam explosion but using a gas that is considered a green, non-toxic, non-flammable, and easy to recover solvent.

The hydrothermal pretreatment consists in using liquid water at high temperatures to hydrolyze hemicellulose, or also cellulose. It is economically and environmentally attractive,

and does not need any chemicals or corrosion-resistant equipment (Batista Meneses et al., 2020). In this thesis GP was submitted to a hydrothermal treatment, since water is considered the "greenest solvent" for use in extraction and hydrolysis processes. It is cheap, non-toxic, easily recycled, non-flammable, available with high purity, and environmentally friendly. Hence hydrothermal pretreatment will be described in more detail in the next sub-section.

The pretreatments referred above lead to the depolymerization of the lignocellulosic matrix. They can be used to hydrolyze partially or completely cellulose and hemicellulose, or to separate the main structural components of lignocellulose. The choice of a suitable pretreatment depends on the material and the desired final application. Drawbacks may include the degradation of target species, high energy consumption, use of large amounts of organic solvents, high costs. The implementation of a sustainable, environmentally friendly technology needs to be balanced with economic feasibility at industrial level.

1.3.1.5 Subcritical water extraction/hydrolysis (hydrothermal pretreatment)

Subcritical and supercritical water have gained attention in recent years, due to the necessity to create sustainable industrial processes that respect green chemistry principles, in particular the use of green solvents. (Marcus, 2018).

The first interest in sub- and supercritical water technology occurred in the late 70s, promoted by the first oil crisis and environmental concerns. Research changed from studying high-pressure steam properties and physicochemical properties of water to focus on the development of alternative fuels, the conversion of biomass and waste treatment. Research on this topic has increased over the years due to the unique properties of water at sub- and supercritical conditions (Brunner, 2009).

Supercritical water is water above its critical point (374 ^{o}C and 221 bar), while subcritical water (SBW), or hot compressed water, is water below the critical point and above the boiling point at ambient pressure (100 ^{o}C and 1 bar). To maintain water in the liquid state, pressure is applied above the water vapor pressure, as represented in **Figure 1.10** (Möller et al., 2011).

The physical and chemical properties of water change significantly with the temperature, influencing its properties as a solvent. Pressure has a negligible influence, being applied essentially to maintain water above the vaporization curve as temperature increases.

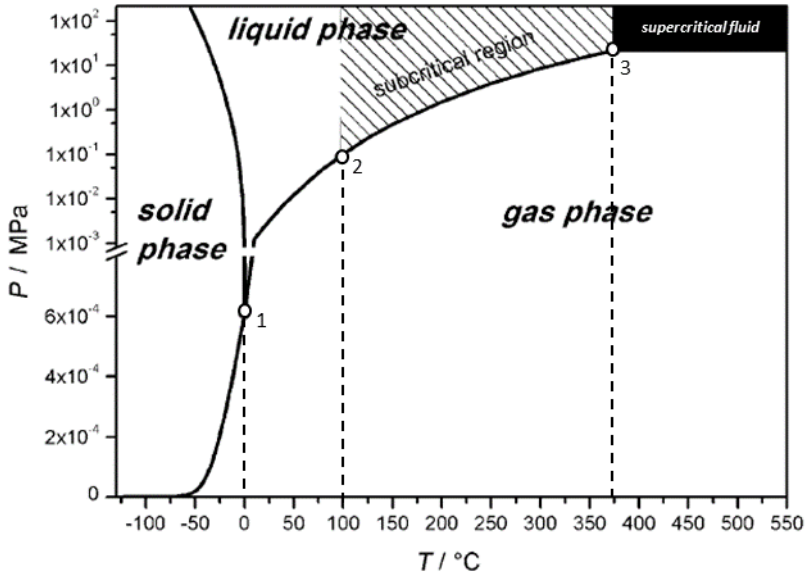

Figure 1.10 - Water phase diagram with the triple point (1), boiling point at ambient pressure (2), critical point (3) and subcritical water region indicated (adapted from Möller et al. (2011)).

Water at ambient conditions is a highly polar solvent, with a high dielectric constant (78.5) due to the hydrogen bonds between the water molecules, making water unsuitable as extraction solvent for non-polar or organic compounds, but adequate to dissolve salts. With the temperature increase, the dielectric constant of water decreases due to the breakage of hydrogen bonds, and water becomes able to solubilize higher amounts of hydrocarbons, and gases such as O_2, N_2, NH_3, CO and CO_2, while inorganic compounds, such as salts, precipitate (Brunner, 2009). Water thus becomes a better solvent for compounds usually extracted with organic solvents. Another advantage of using SBW is the ease of downstream separation, since after cooling down to ambient conditions, the precipitation of those compounds occurs. As illustrated in **Figure 1.11**, the dielectric constant of water can be tuned through changes in temperature to values similar to those of ethanol and methanol, two organic solvents commonly used in extraction processes (Möller et al., 2011; Zhang et al., 2020).

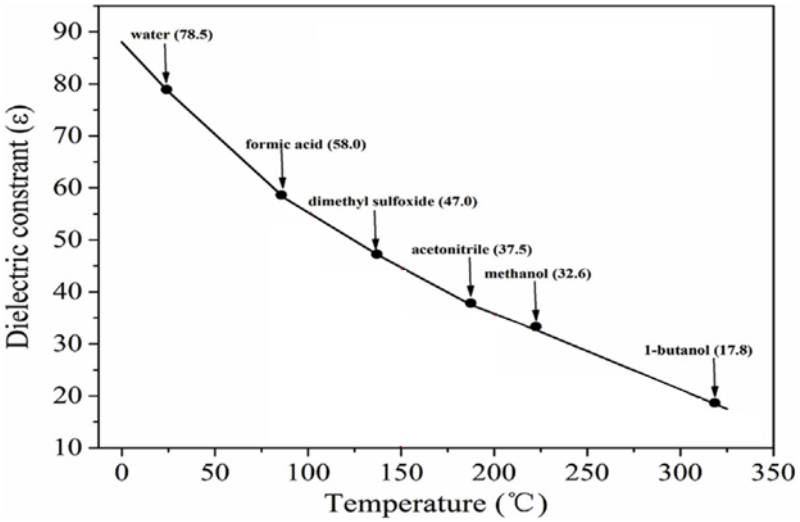

Figure 1.11 – Impact of temperature on the dielectric constant of water at fixed pressure (200 bar), and comparison with the dielectric constant of organic solvents at ambient temperature (adapted from Zhang et al. (2020).

The viscosity and surface tension of water also decrease with the increase of temperature, leading to a faster mass transfer. Additionally, there is an increase of water diffusivity, allowing a better penetration of the matrix particles during extraction processes.

While dielectric constant, viscosity and surface tension are important in extraction processes, the ionic product of water is the main property in hydrolysis reactions. Water dissociation is an endothermic process (Kruse and Dinjus, 2007; Möller et al., 2011). The ionic product (K_w) of water increases considerably with temperature, from the familiar value of 10^{-14} at 25 ºC, to 10^{-11} at 300 ºC, which represents a three orders of magnitude increase. The amounts of H^+ and OH^- ions increase substantially due to water dissociation, favoring ionic reactions since water behaves as an acid/base catalyst. This phenomenon can be exploited in biomass processing for the hydrolysis of lignocellulose. At high temperature, water behaves as an acid catalyst, no addition and later disposal of mineral acid being required.

There are two modes of operation in a SBW process: static, or dynamic. In the static process, water, and the material to be processed are placed inside a (batch) reactor, which is then heated and pressurized to maintain water in the liquid state. During this process a stirrer is used to enhance heat and mass transfer. Remaining solids and liquors are recovered at the end of the process, after cooling down the system. Dynamic SBW extraction is done in a continuous or semi-continuous process. In a semi-continuous process, the material to be treated is placed in

the reactor, and water is pumped continuously. In this type of process, temperature can either be constant, or varied during the assay. In the latter case, different ranges of temperature and hence of water properties allow the recovery of different products. Pressure is usually kept constant. In a continuous process, water and the material are pumped together, and the reaction occurs when water is heated to the desired temperature. This process is similar to the static one, but a larger amount of material can be treated in a shorter time (Cheng et al., 2021).

1.3.1.5.1 Lignocellulose hydrolysis with SBW

1.3.1.5.1.1 Hemicellulose

There are two types of hemicellulose: one easily hydrolyzed and extractable, and another linked to cellulose fibers that can only be obtained upon cellulose hydrolysis (Cocero et al., 2018).

SBW is one of most promising techniques to extract/hydrolyze hemicellulose, at relatively mild conditions, between 160 and 210 ºC. At around 180 ºC, about 60% of hemicellulose can be recovered as monosaccharides, or oligosaccharides (Yu et al., 2008). To obtain higher yields, higher temperatures are needed. However, degradation products may be formed. Another option is the recovery of hemicellulose at low temperature (90 ºC). Yet this requires days to take place, being more time consuming and having higher energy costs (Cocero et al., 2018).

The steps involved in hemicellulose treatment with SBW are hemicellulose cleaving into lower molecular weight oligomers, deacetylation, dissolution, further depolymerization to produce simple sugars, and sugar degradation into e.g. furfural (Cocero et al., 2018; Ruiz et al., 2013).

The cleavage of hemicellulose starts at mild conditions. When the temperature increases, the bonds between sugars break randomly. When the oligomers produced are small enough, they are solubilized, and can be collected. To achieve a high yield of monomeric sugars, temperatures around 180-190 ºC need to be applied. But again, a higher amount of degradation products are formed (Yu et al., 2008). Depending on the final application, this could also be an option. E.g. furfural can be used to produce many chemicals. Another way to control the extent of degradation is to decrease the residence time by increasing the water flow rate (Cocero et al., 2018).

1.3.1.5.1.2 Cellulose

Dissolution and hydrolysis of cellulose in SBW occurs first at the surface of cellulose, in regions containing the amorphous part of the structure. At 250 ºC, it was observed that cellulose is dissolved but not hydrolyzed, resulting in the extraction of high molecular weight products. It was also described that at this temperature, with increasing time, the dissolved cellulose is hydrolyzed to glucose and then into degradation products, if the residence time is too high. Crystalline cellulose is only dissolved above 280 ºC. It only turns into amorphous cellulose at 320-330 ºC, at which complete dissolution takes place (Cocero et al., 2018). It is reported that at 180 ºC, 5% of cellulose is degraded after 5 minutes of residence time, and at 260 ºC the value increases to 80%. Degradation reactions include isomerization, tautomerization, hydration, dehydration, and cyclization, where the main product obtained is 5-HMF. The other products with industrial value are acetic acid, formic acid, lactic acid, and levulinic acid (Möller et al., 2011).

After pretreatment but before complete degradation, cellulose can be submitted to enzymatic hydrolysis that will, more efficiently, produce glucose.

1.3.2 Lignocellulose based materials

Cellulose, the most abundant polysaccharide in nature, can be used in the production of added-value materials after being separated from lignin and hemicellulose. Cellulose is mainly used to produce paper and cardboard. Due to its high crystallinity and stable structure, and as mentioned earlier, cellulose is not soluble in water or common organic solvents, which hinders gel or film formation in its natural state. Hence, cellulose needs to be modified in order to be transformed into a range of useful products (Abdul Khalil et al., 2017). Cellulose acetates are the most common cellulose derivatives used, showing high permeability, solubility in different organic solvents, and biocompatibility. They have a wide range of applications in textiles, filters and membranes, moldings, protective coatings, insulating and fireproofing materials, liquid crystalline displays, controlled drug delivery, wound dressing. Cellulose acetates are also used in photographic and cinematographic films, x-ray films, and microfilms (Ten and Vermerris, 2013). Other cellulose derivates with higher water solubility are hydroxypropyl methylcellulose (HPMC), methylcellulose (MC), hydroxypropyl cellulose (HPC), hydroxyethyl cellulose (HEC),

and carboxymethyl cellulose (CMC), which have similar properties and can have the same applications in the food and pharmaceutical industries (Schantz et al., 2014).

Cellulose nanocrystals (CNC) and cellulose nano whiskers (CNW) can be obtained from the high crystalline cellulose chains, while micro fibrillated cellulose (MFC) and nano fibrillated cellulose (NFC) are obtained from the non-crystalline areas, after a suitable pretreatment. MFC can be used as reinforcing materials in polymer matrices, and after processing into superhydrophobic and oil-repellent aerogels, it can also be used as a packaging material due to its barrier properties. CNC and CNW can also be used as a reinforcing agents for water-soluble and insoluble polymers (Ten and Vermerris, 2013).

Hemicellulose can also be used in the production of polymers. Although it has been less studied than cellulose and lignin for that purpose, interest in exploiting hemicellulose has grown recently and will be studied in this thesis, using hemicellulose oligomers recovered from GP, after pretreatment. Packaging films present a great commercial opportunity in the food industry, since they can provide physical and chemical protection to the food products, due to their flexibility and barrier properties against gases, light, or water. Their oxygen permeability is low, with similar values to petroleum-based materials (Zhao et al., 2020a). On the downside, most hemicellulose films have poor mechanical properties. Some modifications, such as adding a plasticizer, can improve elasticity and barrier properties (Huang et al., 2018). However, this can lead to an increase in hydrophilicity. Another possible modification is blending with a reinforcing agent, such as cellulose derivates, which can improve the mechanical properties of the packaging materials (Nechita et al., 2021; Zhao et al., 2020b). Hydrogels can be formed from galactoglucomannan and from xylan, showing good mechanical properties, viscoelasticity, and water holding capacity. Their main applications are in drug release systems, water purification and wound dressing (Al-Rudainy et al., 2019; Binma-ae et al., 2020; Ten and Vermerris, 2013).

Lignin is an aromatic polymer with different chemical and physical properties from polysaccharides. For many years it was treated as waste during the valorization of cellulose and hemicellulose. However, this view has changed. Lignin and its derivates can have a wide range of applications as a sorbent for metal ions, functional polymer fillers, pharmaceuticals, drug delivery systems, and electrochemically active systems (Yu and Kim, 2020). Lignin-based

composite materials can be applied in sensors, responsive materials, energy storage materials, functional packaging, and biomedical materials. In packaging, lignin can improve mechanical strength, thermal and water stability, and resistance to UV radiation of bioplastics. Lignin can also have a large range of applications in construction, as a cement additive, raw material for polyurethane foams, resins, and bitumen, in asphalt production (Jędrzejczak et al., 2021). Lignin can also be decomposed and used in the production of different chemicals such as vanillin, cycloalkanes, and phenols.

1.3.3 Hemicellulose based prebiotics

The human intestine contains more than 400 species of bacteria, mostly Bifidobacteria and Lactobacilli probiotic bacteria that have health-beneficial effects. These microbes are present in concentrations up to 10^{11}-10^{12} cells/g of luminal contents. Decreasing the proportion between these microorganisms and pathogenic bacteria can cause health issues (Lee et al., 2021; Zhang et al., 2018). For example, long term treatment with antibiotics can cause a disruption in gastrointestinal regular functioning due to the growth of *Candida albicans* and different multiple resistant bacteria, such as *Clostridium difficile*, *Escherichia coli*, *Staphylococcus aureus,* and *Enterococcus* (Kondepudi et al., 2012). The equilibrium can be restored and maintained in two ways. One way is by using probiotics, which are exogenous bacteria that change the intestinal microbiome. Another way is using prebiotics, which were defined by Gibson et al. as nondigestible food ingredients that have a beneficial effect on the host, by selectively stimulating the growth of a limited number of bacterial species in the colon (Gibson, G. R., Roberfroid, 1995; Gibson et al., 2010).

Hemicellulose is a natural source of prebiotics. Prebiotics should be resistant to acidic and enzymatic digestion, absorption in the upper gastrointestinal tract, and should be fermentable by intestine microorganisms while stimulating only the growth of specific bacteria, such as Bifidobacteria and Lactobacilli, which will improve host health and well-being (De Jesus Raposo et al., 2016).

Different hemicellulose-derived carbohydrates, such as fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS) and lactulose, are already in the market, while others such as xylooligosaccharides (XOS), and arabinoxylans, are emerging candidates as prebiotics since

they present the same, or better, properties when compared with the more well-established prebiotics in the market (Figueroa-González et al., 2019; Mueller et al., 2017). XOS have recognized potential for reducing the risk of colon cancer, and for having a cytotoxic effect on human leukemia cells. Furthermore, when compared with FOS, XOS are more stable at lower pH and at pasteurization and sterilization temperatures, making them more suitable for food applications (Otieno and Ahring, 2012).

1.3.4 Phenolic compounds

It has been referred earlier that phenolic compounds are among the most valuable nonstructural components of agro-industrial by-products, including GP. Phenolics-rich extracts have the potential to be used in food, cosmetic and pharmaceutical industries due to their bioactivity. As an antioxidant, they have been studied for the inhibition of lipid oxidation and warmed-over flavor in food systems.

In particular, GP extracts were able to reduce rancid flavor, from primary and secondary lipid oxidation products in different food products, such as raw and cooked beef, turkey, or frozen fish, maintaining the pH, water activity, and products yield (Yu and Ahmedna, 2013). Antimicrobial activity is another important property. GP extracts showed activity against pathogenic and spoilage bacteria, such as *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus*, Salmonella Enteritidis, and Salmonella Typhimurium (Beres et al., 2017). Resveratrol has also been reported as a strong fungicide against *Candida albicans, Saccharomyces cerevisiae,* and *Trichosporon beigelli.* These reports show that GP extracts have the potential to be used as a food preservative and also in medicine due to their capacity to avoid the growth of pathogenic bacteria and lipid oxidation (Yu and Ahmedna, 2013). In cosmetics, GP phenolics can be used as an anti-aging agent, since they can inhibit enzymes related to skin aging, such as collagenase and elastase. The more promising results were shown by water soluble compounds with low molecular weight, mainly gallic acid (Beres et al., 2017).

Prevention of cardiovascular diseases, usually associated with moderate wine consumption, can also be prevented by phenolic compounds. Cardiovascular disease is caused by modifications in fatty acid metabolism and excessive lipid peroxidation of low-density lipoprotein (LDL), which generates products that increase platelet aggregation and cause artery

blockage and thrombosis. GP extracts showed the capacity to inhibit LDL oxidation, reducing the risk of heart disease (Yu and Ahmedna, 2013).

Phenolics also showed inhibitory activity against acrylamide formation. Acrylamide is a carcinogen that can be formed during food heating through the Maillard reaction that occurs between sugars and amino acids in foods. The studies showed a 90% decrease in acrylamide formation when using GP phenolic extracts (Bordiga et al., 2019).

Bioactive compounds from grape pomace have high-value and are already on the market. Some examples are food supplements with resveratrol, 100 Natural®, Nature's Way®, Maximum Strength®, GrapeSeedRich®, as well as cosmetic products such as day and night cream and face serum from Pure Super Grape®, and mattifying fluid, anti-wrinkle and protect fluid from Caudalie® (Beres et al., 2017).

1.3.4.1 Methods of extraction of phenolic compounds

SBW extraction has been shown to be very efficient for the extraction of phenolic compounds. Before describing SBW extraction, other methods available will be reviewed.

Solid-liquid extraction (SLE) is a very common extraction technique, widely used for the recovery of phenolic compounds from GP. SLE efficiency depends on solvent type, temperature, particle size, and time of extraction. The solvent type represents the main factor, and hydroalcoholic solutions are usually used due to their considerable good extraction efficiency. The most used solvents are ethanol, methanol, acetone, and water. Methanol presents the best extraction yields, especially mixed with water. The solvent efficiency also depends on the target compounds. For instance, acetone/water has been indicated as good solvent to extract procyanidins, while ethanol/water is reported to be better to extract catechins and procyanidins, and methanol for the extraction of catechins, epicatechins, and epigallocatechins. Temperature and time also are important to achieve high recovery yields with low energy costs. Higher temperatures, up to 50-60 ^{o}C, will improve solubility and diffusion while avoiding thermal degradation of the compounds to be recovered.

Notwithstanding, SLE is not an environmentally sustainable process, since it uses large amounts of solvents and is time consuming. The use of ultrasound radiation instead of the common mechanical stirring helps to improve extraction efficiency, by increasing mass transfer and

diffusion of the solvent and consequently reducing extraction time, without the use of high temperatures. The efficiency of ultrasounds can be explained also by fragmentation of the matrix by the radiation (Fontana et al., 2013). Overall, due to their environmentally friendly character and low cost, water/ethanol mixtures can be used in association with ultrasounds to reduce time and temperature of an extraction process.

Accelerated Solvent Extraction (ASE) uses the conventional solvents at high temperatures and pressures, enhancing the extraction of phenolics. The high pressure improves the solvent penetration into the matrix, while the solubility of the compounds increases at high temperatures. The temperature also reduces viscosity and surface tension, which leads to a faster mass transfer. ASE can lead to higher recovery yields in shorter times, but it needs more expensive equipment and has a considerable energy cost that needs to be taken into account (Beres et al., 2017; Fontana et al., 2013).

To avoid the use of organic solvents, decrease time and temperature of the process, as well as increase mass transfer and extraction yields, other techniques have been studied, such as Pulsed Electric Fields (PEF) treatment. This consists in placing the matrix between two electrodes, and exposure to the electric field creating pores (electroporation) in the plant cell membranes. This non-thermal process increases cell membrane permeability and consequently improves mass transfer. This method has shown to improve the SLE of phenolic compounds, mainly anthocyanins from RWGP. This equipment is already used by food industries to scale-up extraction processes and the pretreatment of food matrices. Pulsed Ohmic Heating (POH) consists in the combination of PEF treatment with moderate temperatures (20-50 ^{o}C) to improve the extraction process (Barba et al., 2016).

High Voltage Electrical Discharges (HVED) is another electrical treatment, but in this case high voltages are applied to accelerate electrons and excite water molecules, creating a flood of electrons that propagates from the positive to the negative electrode leading to an electrical breakdown. Then, high-amplitude shock waves, bubble cavitation and liquid turbulence results in particle fragmentation and consequent cell structure damage, which will increase the extraction of compounds to water. If combined with freezing or high temperatures, the process can be enhanced, although studies showed that the scale-up of the process brings high energy costs to the process (Barba et al., 2016; Fontana et al., 2013).

Microwave Assisted Extraction (MAE) is another green process that can reduce extraction time and solvent consumption. It consists in the use of electromagnetic energy to warm up the water inside the matrix, which evaporates, creating pressure that eventually leads to cell membrane disruption, allowing the extraction of the target compounds. Studies on MAE of phenolics from GP showed extremely high efficiency compared with conventional SLE, with a reduction of extraction time from 5 h to 5 min, and additionally higher yield due to the extraction of compounds bound to the matrix (Barba et al., 2016; Bodoira and Maestri, 2020).

Alternative solvents for SLE are deep eutectic systems (DES). DES, and especially DES made up of natural components such as choline chloride, urea, ethylene glycol, glycerol, alcohols, amino acids, carboxylic acids or sugars, are green solvents, have a low price, are easy to prepare, are biodegradable, and have no or very low toxicity. Properties such as freezing point, conductivity, density, viscosity, and polarity depends on DES composition. The DES ability to donate and accept protons and electrons can improve extraction efficiency. The high viscosity of many DES can have a negative impact on the extraction efficiency, which in certain cases can be counteracted with the addition of water (Paiva et al., 2014; Panzella et al., 2020).

1.3.4.2 SBW extraction

Extraction using SBW consists in the transfer of solutes from the matrix to the solvent by diffusion, partitioning equilibrium, and convection. To avoid oxidation most of the processes are performed under inert atmosphere, using gases such as nitrogen to purge the system. This method altogether by-passes the use of organic solvents. Extraction conditions may change depending on the composition of the matrix used and the amount and type of phenolics present. The extraction efficiency depends on different parameters, such as pressure, temperature, solvent ratio, particle size, extraction time, mixing, and flow rate in dynamic extraction (Essien et al., 2020).

Pressure, as referred earlier, has low impact on extraction, being used to maintain water above the vaporization curve when temperature increases. In any case, higher pressure can enhance extraction by forcing water through the matrix pores.

Temperature plays the most important role since the increase of temperature and the decrease of viscosity and surface tension cause the reduction of cohesive (solute-solute) and adhesive

(solute-matrix) intermolecular interactions. Also, as mentioned earlier, the decrease in the dielectric constant of water results in a higher solubility of less polar compounds.

The solvent ratio (amount of solvent divided by amount of material to be treated) affects the solubility of phenolics, since increasing the amount of solvent will improve the yield of extraction. Flow rate is only relevant in the dynamic mode and has a positive impact on extraction yields since fresh solvent is always in contact with the matrix. Higher flow rates (lower residence times) can also avoid thermal degradation of the compounds. However, more diluted liquors/extracts are produced. Smaller particle sizes will affect positively the extraction process, by providing comparatively higher surface area and by facilitating diffusion (Essien et al., 2020).

The use of SBW in the extraction of phenolic compounds has been applied mainly to fruit and other food wastes (Aliakbarian et al., 2012; Gil-Martín et al., 2022; Meneses et al., 2013). E.g. SBW extraction at 210 ^{o}C allowed a recovery of 2.6 g/100 g of phenolics from WWGP, which is twice the amount obtained using conventional solid-liquid extraction (Pedras et al., 2017). Mikucka et al. (2022) worked with distillery silage, obtaining the highest extraction yield of phenolic acids and flavonoids at 140 ^{o}C and 200 ^{o}C, respectively. In another study, Vladić et al. (2020) found that for SBW extraction from pomegranate peel, 130 ^{o}C was the optimal temperature to obtain a high yield of extraction, and a low amount of HMF. SBW extraction from papaya seeds was successfully performed at 150 ^{o}C, yielding extracts with a high antioxidant potential (Gonçalves Rodrigues et al., 2019).

1.3.5 C6 and C5 sugars

Glucose and xylose, the most abundant C6 and C5 sugars resulting from the hydrolysis of cellulose (C6) and hemicellulose (C5 and C6), can have a wide range of applications as a carbon source for the microbial production of high-value products, or to obtain important chemicals through chemical conversion.

1.3.5.1 Bioenergy

Bioethanol is sold in several countries mixed with gasoline. It is essentially produced from sucrose and starch, which are easily hydrolyzed and fermented to obtain ethanol. However, the process is not cost-efficient, is still more expensive than the fossil competitors, and usually

needs dedicated crops. On the other hand, 2nd generation bioethanol is produced through the fermentation of sugars obtained from lignocellulose. This is a more complex process, but it can be more profitable due to the low-cost available feedstock. Additionally bioethanol can be co-produced with other valuable chemicals in a biorefinery platform (Chrysikou et al., 2018).

1.3.5.2 Platform chemicals

Platform chemicals are defined as chemicals that can be used in the production of different added-value products. These chemicals can be obtained from the polysaccharides present in biomass. The Department of Energy of the United States defined 12 platform chemicals that can be obtained from the lignocellulosic structure, with a potential to be a sustainable alternative to petroleum-based chemicals (Takkellapati et al., 2018). In addition to ethanol, two of the most common chemicals derived from lignocellulose are furfural and 5-HMF, which are formed by degradation of C5 and C6 sugars respectively, most commonly through acid catalysis. Furfural can replace formaldehyde and is mostly converted into furfuryl alcohol, but also converted into succinic acid or levulinic acid, with main applications in plastics, pharmaceutical and agrochemical industries, in adhesives and flavor enhancers (Takkellapati et al., 2018).

Originating in C6 sugars, mainly glucose, 5-HMF can be converted into polyester building blocks, or 1,6-hexanediol (1,6-HDO), which is used in the production of polyurethanes for coating, elastomers, and adhesives, as well as in different polymer production, when converted into 1,6-heaxanediamine and ε-caprolactone (Takkellapati et al., 2018). Another compound produced directly from sugars or HMF is 2,5-furandicarboxylic acid (FDCA), which finds use in the synthesis of polyesters, polyamides and plasticizers. These polymers have similar properties to their petroleum-based counterparts.

Sorbitol is the most used sweetener, with an annual production of 800,000 tons. It has a wide range of applications in food, beverages, drugs, cosmetics and chemicals, and is produced from glucose. Sorbitol can be used in ascorbic acid production, and converted into several alcohols, such as propylene glycol, ethylene glycol, ethanol, or methanol. It can also be used in the production of biodegradable polymers and biocomposites. Xylitol is also a sweetener, obtained from xylose. It can be used as a sugar substitute by diabetic people, since it does not depend

on insulin to be metabolized (Takkellapati et al., 2018). Xylitol can also be used to produce packaging films or hydrogels (Zhao et al., 2020a).

1.3.5.3 C5 and C6 sugars as carbon source for microbial processes

A large amount of chemicals and materials can be produced by biochemical transformation of C6 and C5 sugars. Biological conversion appears as the most flexible biorefinery process to transform biomass into marketable products. Glucose is the preferred carbon source of microorganisms, being of high-value to biorefineries.

Biopolymers can be produced by different microorganisms such as bacteria, fungi, and yeast, being mostly produced by bacteria or fungi at the industrial level. For instance, dextran, gellan, and xanthan can be produced from agro-industrial biomass using bacteria, and pullulan from hemicellulose sugars using fungi (Philippini et al., 2020).

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxy acids, accumulated by different groups of bacteria as carbon sources. They can have numerous applications in packaging materials, bags, bottles, drug delivery carriers, cups, diapers, films and medical devices, such as cardiovascular stents (Ortega et al., 2021; Philippini et al., 2020). The interest in PHAs as alternative plastics relies on their biobased nature, biodegradability, and versatility (Meneses et al., 2021; Philippini et al., 2020). Bacterial nanocellulose (BNC) is another biopolymer with great interest since it has a low production cost, good mechanical properties, is biocompatible and biodegradable. BNC is similar to plant cellulose, but with high purity and strength, having higher crystallinity and polymerization degree, better liquid absorbent capacity and mechanical properties, making it a more interesting option for numerous applications (Ortega et al., 2021).

Other products other than biopolymers can be produced by microorganisms from agro-industrial wastes, such as antibiotics, enzymes, and pigments, decreasing the production costs of these high-value products. Antibiotics are of extreme importance since they can eliminate harmful microorganisms at low concentrations. Enzymes produced by microbes are one of the most profitable products, with a market value of 5.5 billion dollars in 2018 and with a projection to achieve 7 billion in 2023 (Batista Meneses et al., 2020). Biopigments, such as chlorophylls, melanin, carotenes and xanthophylls, can be directly extracted from plants or produced by microbes. These compounds can also be chemically synthesized, but with associated toxicity

and health effects, while biopigments are non-toxic and safe (Yaashikaa et al., 2022). The downside is that biopigments cost almost three times as much as their synthetic counterparts. Nevertheless, demand for biopigments is increasing with the growing interest for natural and safe products (Philippini et al., 2020).

Other products of interest are microbial oil – microbial lipids - and biosurfactants, which are also the focus of this thesis and will be mentioned in greater detail in the next sub-section.

1.3.5.3.1 Microbial oil

Microbial oil can be obtained from oleaginous microorganisms. These are a group of microorganisms that can accumulate over 20% of lipids as cell weight. This group includes fungi, yeast, and algae, as well as some autotrophic and heterotrophic bacteria that accumulate essentially triglycerides (TAGs) and fatty acids (FAs). The first reported use of microorganisms for lipid production dates from 1870, but with no clear economical advantage compared with plant lipids. More recently, due to the implementation of the concepts of bioeconomy and circular economy, research on the topic took off. Fermentative processes do not require extensive land use and human resources when compared with plant cultivation, and are not dependent on location and climate, with the additional easy scale-up (Guerfali et al., 2019). Since microbial oil from different oleaginous species presents a FA profile very similar to that of vegetable oils, microbial oil is being considered an alternative to lipids currently produced by dedicated crops (Caporusso et al., 2021).

Some oleaginous yeasts, in particular, can accumulate large amounts of lipids, in addition to being a source of other valuable compounds such as enzymes, biosurfactants, antimicrobial molecules, pigments, exopolysaccharides, and organic acids (Guerfali et al., 2019). The main advantages of yeasts in a biorefinery process, compared to fungi, microalgae and bacteria, are their higher biomass production, the ability to grow in different types of carbon sources, and easy cultivation in large fermenters (Caporusso et al., 2021). Yeasts are widely distributed in nature, are found in soil and water, have the capability to survive in extreme environments, such as sea depths, low temperatures, and scarce oxygen, or in contaminated environments. There are about 1,500 known species from 100 genera, and 30 of them are able to accumulate lipids. The most studied genera are *Yarrowia, Rhodotorula (Rhodosporidium), Lipomyces,*

Cryptococcus and Trichosporon. Of these, *Yarrowia lipolytica* is the most studied species due to its unique properties as host for different industrial processes such as dietary supplements and nutraceuticals production, and as a model to study lipid synthesis and accumulation by microorganisms (Caporusso et al., 2021). Other species have also been studied, such as *Rhodosporidium toruloides, Lipomyces starkeyi, Trichosporon fermentans, Trichosporon pullulan, Rhodotorula glutinis, Trichosporon capitatum and Cryptococcus curvatus,* which can accumulate over 50% of lipids (Ahmad et al., 2019).

Lipid production in microorganisms can occur via *de novo* or *ex novo* lipid synthesis processes. In *de novo* processes, lipid accumulation occurs after depletion of an essential nutrient during fermentation. The oleaginous yeasts use the excess carbon to produce oil, in stress conditions created by the absence of nitrogen, or less commonly phosphorus. The stress conditions lead to an accumulation of citric acid in the mitochondria, which is secreted to the cytoplasm, where it is converted to acetyl coenzyme A (acetyl-CoA), the fatty acid synthesis precursor, and oxaloacetate by ATP citrate lyase. In the *ex novo* process, lipid accumulation occurs in the presence of fats or other hydrophobic compounds that are used as the carbon source, with lipids being synthesized even in the presence of nitrogen. The nitrogen limitation is the most used stress condition for industrial microbial oil accumulation, the main strategy to enhance oil production being to increase the carbon to nitrogen ratio (C/N). Other strategies are to avoid lipid turnover or catabolism, which usually happens when cells need carbon for maintenance or growth and the extracellular sources are depleted (Ahmad et al., 2019; Maina et al., 2017). This metabolism is specific to oleaginous microorganisms since non-oleaginous microorganisms in similar stress conditions will channel the excess carbon to the production and storage of polysaccharides (Ratledge, 2004).

Microbial oil is also called single-cell oil (SCOs). Some species, under specific conditions, can accumulate SCO up to 70% of cell weight. SCOs are mainly composed of TAGs (80-90%), with low amounts of phospholipids, sphingolipids, sterols, and free fatty acids.

The TAGs produced are composed of long-chain fatty acid moieties, mainly C16 and C18, that can be unsaturated, monounsaturated (MUFA), or polyunsaturated (PUFA). The most abundant fatty acids accumulated are palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), with lower amounts of myristic acid (C14:0) and

linolenic acid (C18:3). Genetic engineering has led to the production of higher amounts of PUFA and long-chain fatty acids, such as arachidic acid (C20:0), arachidonic acid (C20:4), behenic acid (C22:0) and lignoceric acid (C24:0) (Caporusso et al., 2021).

Although the fatty acid composition of SCO is like the one found in plant oil, the microbial process is still not economically sustainable. The cost of plant oil is approximately $1.5-3/kg, while SCO from yeast costs $5.5/kg. Using zero cost waste (glucose at cost zero) and by-products streams, the value above decreases to $3.4/kg. Hence, to make SCO competitive and decrease production costs, an integrated biorefinery concept is being studied (Guerfali et al., 2019; Probst et al., 2016), for instance involving the co-production of other metabolites such as enzymes (cellulase and lipase), biosurfactants (glycolipids) and carotenoids (Guerfali et al., 2019).

Rhodotorula babjevae is an oleaginous red yeast, and one of the highest lipid producers (Guerfali et al., 2019; Sitepu et al., 2013). During lipids accumulation, R. *babjevae* synthesizes carotenoids and secretes biosurfactants, which are more valuable products than microbial lipids just for biodiesel production (Garay et al., 2018, 2017a; Sperstad et al., 2006). This is particularly relevant in this thesis and will be approached in more detail in the next sub-chapter.

1.3.5.3.2 Carotenoids

Carotenoids are natural lipid-soluble pigments with different colors from yellow to red, being also precursors of vitamin A. These compounds act as membrane-protective antioxidants, scavenging O_2 and peroxyl radicals. In plants and algae, carotenoid pigments are synthesized due to their light-harvesting capacity. In non-photosynthetic systems, such as fungi and yeasts, carotenoids protect against light and air (Buzzini et al., 2007a; Mata-Gómez et al., 2014).

Carotenoids comprise more than 600 different structures. They are terpenoid pigments with 40 carbon atoms and are mainly soluble in non-polar solvents. They are divided into two groups, namely carotenes, and xanthophylls (Kim et al., 2012). Carotenes contain only carbon and hydrogen, while xanthophylls also contain oxygen. The three main carotenoids found in *R. babjevae* are carotenes, namely torulene, torularhodin, and β-carotene (Figure 1.12), which impart red color to the yeast (Kristian and Synnøve, 2006).

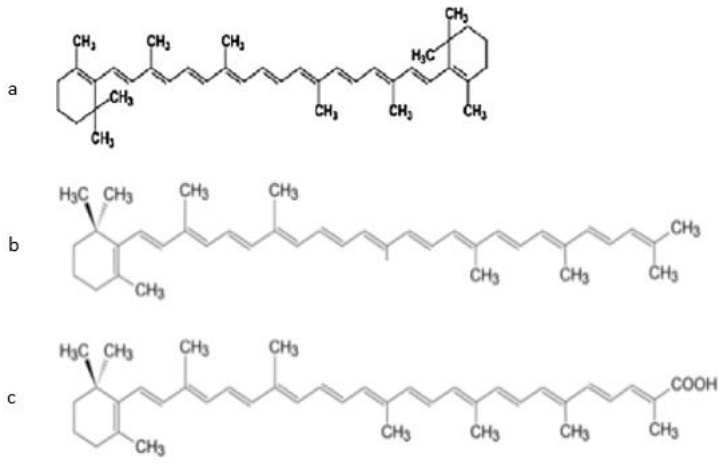

Figure 1.12 - Molecular structures of β-carotene (a), torularhodin (b), and torulene (c) (adapted from Mata-Gómez et al. (2014) and Zoz et al. (2015)).

Carotenoids can be used in the food industry as coloring agents, and in the pharmaceutical industry as additives to cosmetics. The interest in these compounds is not only related to the fact that they are natural pigments, but also their bioactive properties. Carotenoids are known for their antioxidant activity, which is related to the prevention of degenerative diseases such as cancer, cardiovascular diseases, macular degeneration, and cataract, being considered a high value product with a growing market, reaching 2 billion USD in 2022 (McWilliams, 2018). Natural carotenoids are similar to synthetic ones, but with some differences at molecular level that give them higher health benefits. The industrial production of natural carotenoids can be done through biotechnological processes, using microorganisms, or by extraction from plants. Nevertheless, the production of the well-known carotenoid β-carotene from natural sources only accounts for 2% of the total produced (Mata-Gómez et al., 2014). Carotenoids from vegetable sources cannot be produced all year since that depends on seasonal and geographic variabilities. On the other hand, the chemical synthesis of these compounds generates hazardous wastes, which can cause environmental pollution. Microbial production, besides being safe, has the advantage of using low-cost carbon sources, which can reduce the production cost, making microbial processes more attractive to replace the conventional ones.

1.3.5.3.3 Polyol Esters of Fatty Acids

Studies by Garay et al. report the secretion of glycolipids as an extracellular metabolite by *R. babjevae* simultaneously with lipid accumulation (Garay et al., 2017a). Glycolipids are amphiphilic organic molecules produced by different microorganisms with distinct surfactant properties. Two glycolipids biosurfactants already available in the market are sophorolipids, and rhamnolipids, secreted by the yeast *Starmerella bombicola* and the bacteria *Pseudomonas,* respectively (Garay et al., 2017a). Previous studies suggest that polyol esters of fatty acids (PEFAs) can have biotechnological applications since their physicochemical properties give them the potential to be employed as biosurfactants. Biosurfactants are used in industrial and household cleaning products, cosmetics, lubricants, adhesives, agrochemicals, mining, petroleum extraction and cleanup, and in pulp and paper industries, like the usual surfactants derived from petroleum (Garay et al., 2017b). Biosurfactants can replace these surfactants due to their green nature, having a more sustainable production, low toxicity and high biodegradability, which make them also suitable for food, cosmetic, and pharmaceutical applications (Shakeri et al., 2020).

The glycolipids secreted by *R. babjevae* are called PEFAs (Figure 1.13). The main difference of PEFAs from other glycolipids, such as sophorolipids or rhamnolipids, is that the polar moiety in the hydrophilic head is a polyol, instead of the usual carbohydrate. Nineteen different molecules have been identified already. In addition to a polyol head group, PEFAs contain a fatty acid chain. The two main polyols present in PEFA are D-arabitol, and D-mannitol, while the fatty acids are usually C16, and C18, but smaller amounts of C14 and C20 can also be found. PEFA production profile depends most on the strain and less on the growth conditions and the carbon source. *R. babjevae* produces a mixture of PEFAs containing D-arabitol and D-mannitol. Higher amounts of D-arabitol are obtained when *R. babjevae* is cultivated on glucose. When grown on sugar cane molasses, essentially PEFAs with D-mannitol head groups are produced, although this varies among strains of the same specie (Garay et al., 2018).

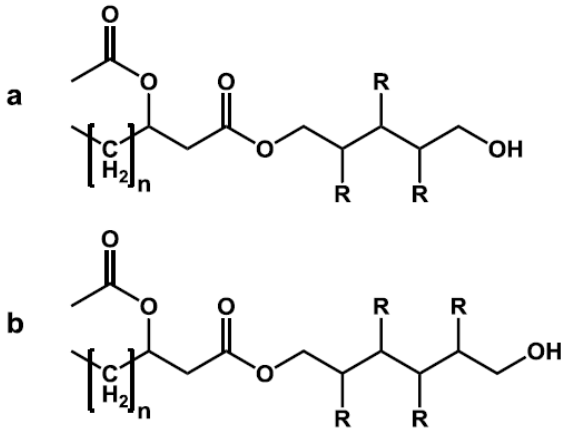

Figure 1.13 - PEFAs structures with D-arabitol (a) and D-mannitol (b) head groups. R can be acetyl or hydroxyl groups, and n is the number of carbon atoms on the fatty acid chain, which can be 8, 10, 12 or 14. Adapted from Garay et al. (2017a).

PEFAs have antifoaming activity, like commercial antifoaming agents used in the brewery industry, and have the capacity to decrease the surface tension of water, reaching values of 30.4 mN/m, lower than the one found for sophorolipids (33-38 mN/m). Studies about their antibacterial and anticancer activities are still lacking. However, similarities with other glycolipids suggest that PEFAs can also exhibit this type of bioactivities (Garay et al., 2018).

Since production costs are still the main barrier to the implementation of microbial processes, *R. babjevae* can be a suitable yeast. The co-production of carotenoid-rich SCOs and glycolipids, associated with the use of alternative carbon sources from wastes streams, such as the GP in this thesis, can be the key to a process that can be sustainable and economically appealing, with the production of multiple high value products and the use of renewable resources, meeting the goals of circular bioeconomy.

1.4 Aims and structure of the thesis

The main goal of this thesis was to develop a green and sustainable process for the complete valorization of grape pomace, a by-product of the winemaking industry, within the scope of biorefinery and circular bioeconomy. Focus was given to the development of a cascade process to obtain different added-value products from RWGP.

Since RWGP is essentially composed of lignocellulosic material, the main objective of this work was to use SBW, an environmentally friendly solvent, for its fractionation into cellulose, hemicellulose and lignin, while additionally recovering phenolic compounds.

For the fractionation, the properties of water, such as dielectric constant and ionic product, were manipulated by increasing the temperature to obtain differentiated extracts. The use of a semi-continuous reactor, and implementation of a stepwise temperature increase, can allow the extraction of phenolic compounds and soluble sugars at lower temperatures (up to 130 ^{o}C), and the recovery of hemicellulose oligosaccharides (up to 190 ^{o}C).

For the separation of cellulose and lignin, two approaches were studied: the solubilization of lignin through a modified organosolv treatment using CO_2, and cellulose hydrolysis with SBW at higher temperatures (up to 280 ^{o}C), in both batch and semi-continuous reactors. Both strategies can lead to the concentration, in the remaining solid residue, of the component not targeted in the treatment.

To add value to the liquors/extracts obtained during the process, applications were studied for some of the GP components recovered. The phenolic-rich extracts were evaluated in terms of their antioxidant and antibacterial activities, to assess their potential for incorporation in cosmetic formulations or food supplements.

The hemicellulose-rich extracts recovered were studied as a source of prebiotics via determination of their prebiotic index and prebiotic activity score, to assess their potential in comparison to commercial prebiotics. Since most of the hemicellulose is recovered as oligosaccharides, those extracts were also tested in the preparation of hemicellulose-based films that can be used for packaging or food coating.

The abundant, immediately available, water-soluble sugars (monosaccharides) of non-fermented white wine grape pomace (WWGP) were extracted through a simple solid-liquid process, with water at ambient temperature. These sugars were studied as alternative carbon source for yeast growth, more specifically *Rhodotorula babjevae*, an oleaginous yeast capable of accumulating lipids and carotenoids, at the same time secreting glycolipids, namely polyol esters of fatty acid (PEFA).

Overall, this thesis studied an approach to obtain different compounds from GP, using green technologies. Additionally, applications of these compounds were also looked at, to add value to this abundant agro-industrial residue.

2

FRACTIONATION OF RED WINE GRAPE POMACE

The results in sub-sections 2.3.1 and 2.3.2 of this chapter have been published as: **Pedras, B.M.**, Regalin, G., Sá-Ngueira, I., Simões, P., Paiva, A., Barreiros, S. Fractionation of red wine grape pomace by subcritical water extraction/hydrolysis. J. of Supercritical Fluids. 160 (2020), 104793.

2.1 Introduction

Grapes are one of the most abundant crops in the world, with a worldwide production of ca. 78 million ton in 2018. From these, 57% were used in winemaking (OIV, 2019). Grape pomace is the by-product generated in the latter process, accounting for ca. 15% of the total grape input, consisting in grape seeds, skin and pulp. In red winemaking, the grape juice is fermented together with the pomace, making red wine grape pomace (RWGP) poor in soluble sugars. Its carbohydrate content appears mostly in the form of structural cellulose and hemicellulose. RWGP has also considerable amounts of lignin and phenolic compounds (Bordiga et al., 2019).

Biomass extractives are commonly obtained with a simple solid liquid extraction, using water or less polar organic, or mixed solvents. For the extraction of carbohydrates, acid hydrolysis is one of the most used methods due to its efficiency. Both methods present drawbacks since both require the use of organic solvents or hazardous chemicals.

Subcritical water (SBW) treatment can be suitable for the fractionation of RWGP into different useful streams. As temperatures increases, water can progressively remove extractives, such as phenolics, proteins and soluble sugars, dissolve hemicellulose components, and decompose cellulose (Cocero et al., 2018; Yedro et al., 2014; Yu et al., 2008).

The main objective in this chapter was to develop a process using mostly SBW, to obtain different RWGP fractions, rich in phenolic compounds, hemicellulose sugars, glucose from cellulose, and lignin. First, using a semi-continuous reactor, phenolics (up to 130 ^{o}C) and hemicellulose carbohydrates (130-190 ^{o}C) were isolated, in extracts with different properties. Then the work was focused on the separation of cellulose and lignin, isolating both by (a) removing lignin and leaving cellulose as residue, using a modified organosolv method, or the inverse process, by (b) removing cellulose and leaving lignin as residue, using SBW at higher temperatures (230-280 ^{o}C).

2.2 Material and methods

2.2.1 Materials

Red wine grape pomace (RWGP) was kindly provided by the Portuguese wine producer Esporão (https://www.esporao.com/), from grapes growth in the Alentejo region, which were destemmed before pressing. Grape pomace collected on a given afternoon was received the following morning and was immediately separated into ca. 600 g portions that were placed in plastic bags and stored at -18 ºC. As needed, the contents of each bag were frozen with liquid nitrogen and lyophilized for 3 days for water removal. The lyophilized powder obtained was milled to ca. 2 mm particle size (RWGP) using an IKA tube mill C S000, well mixed, and stored in plastic bags at -18 ºC. The water content of RWGP was determined gravimetrically, after keeping the material in an oven at 105 ºC overnight.

Phenol (99%), Folin & Ciocalteu's phenol reagent, D-glucose, D-fructose, D-galactose, D-xylose, D-mannose,L-fucose, L-arabinose, gallic acid, syringic acid, vanillic acid, quercetin, catechin and methanol were from Sigma-Aldrich. So were cellulose (C6663, fibers, long; used to determine enzyme activity), cellulase from *Trichoderma reesei* (Celluclast 1.5 L; 135 U/mL; tested on cellulose) and cellulase, enzyme blend (Cellic CTec2; 51 U/mL; tested on cellulose). Sulfuric acid, *n*-hexane, ethanol, were from Carlo Erba, and citric acid and sodium citrate were from Pronalab. All other reagents were of high grade and purchased from available suppliers.

2.2.2 Chemical characterization of RWGP

This characterization was performed as previously reported for WWGP (Pedras et al., 2017).

2.2.2.1 Protein and ash content

The nitrogen content of RWGP was determined by elemental analysis, and converted to protein content by using a factor of 6.25 (Hames et al., 2008). Ash content was quantified by using a muffle furnace. Briefly, 0.8 g of RWGP were weighed in a porcelain crucible and placed in a muffle at 550 ºC. After 6 hours the crucible was removed and left to cool down in a desiccator. Ash content was determined through weight difference (Sluiter et al., 2008a).

2.2.2.2 Phenolic compounds extraction

Phenolic compounds were extracted with a (60:40, v/v) methanol:water solution. Briefly, to 1 g of RWGP, 40 mL of solvent were added, and the mixture was incubated at 60 ºC for 90 min, under constant magnetic stirring (150 rpm). At the end of that period, the mixture was filtered and the solution was used for phenolics quantification (Pedras et al., 2019).

2.2.2.3 Lipids, carbohydrates and lignin determination

Prior to carbohydrate analysis, fat content was determined through Soxhlet extraction of ca. 2 g of RWGP with 65 mL of *n*-hexane, for 4 h. The residue was separated from the solution and dried overnight at 40 ºC, to remove the solvent, after which it was weighed. *n*-hexane was removed from the solution through evaporation under a nitrogen stream, and the remaining oil was weighed (Pedras et al., 2017).

To quantify soluble carbohydrates, 0.8 g of defatted RWGP (def-RWGP) were submitted to extraction with 40 mL of an (80:20, v/v) ethanol:water solution, in an ultrasonic bath for 15 min, followed by centrifugation (9,000 *g*, 10 min, 4 ºC). The process was repeated three times. The three supernatants were combined, and ethanol was evaporated at 50 ºC, under vacuum, in a rotary evaporator. The remaining solution was diluted with 80 mL of water and used for carbohydrates analysis. The solid that did not dissolve in the ethanol:water solution was dried overnight, at 40 ºC (Deng et al., 2011).

To quantify structural carbohydrates, a 2-step hydrolysis with sulfuric acid was performed to the def-RWGP after removal of soluble sugars. Briefly, 0.3 g of this residue was incubated, at 30 ºC, under stirring, with 3 mL of 72% (w/w) H_2SO_4, for 1 h. After this period the mixture was diluted to 4% (w/w) of H_2SO_4 by adding 84 mL of distilled water, and incubated at 121 ºC in a silicone bath, under stirring, for 1 h. The mixture was then filtered and the resulting supernatant was used for carbohydrate quantification, while the solid was washed, dried overnight at 105 ºC, and used to calculate the amount of Klason lignin, by subtracting ash and protein content to the dry residue weight (Sluiter et al., 2008b).

2.2.3 SBW semi-continuous apparatus and experimental conditions

The SBW semi-continuous apparatus used is represented in Figure 2.1. Briefly, a 270 mL HiP reactor, placed in an electric oven, containing a known amount of RWGP (in the range 30−60 g), was submitted to a continuous flow of water at controlled pressure and temperature, using a KNAUER 40 preparative pump 1800 coupled to a RHEONIK RHE 01.03 unit. Before reaching the reactor, water is heated by a heating wire connected to a temperature controller. Pressure is measured with a pressure indicator. The temperature of the liquor exiting the reactor is monitored, before being cooled down by a heat exchanger. The pressure of the system is controlled by a Tescom back pressure regulator (BPR). The liquor was continuously collected for analysis.

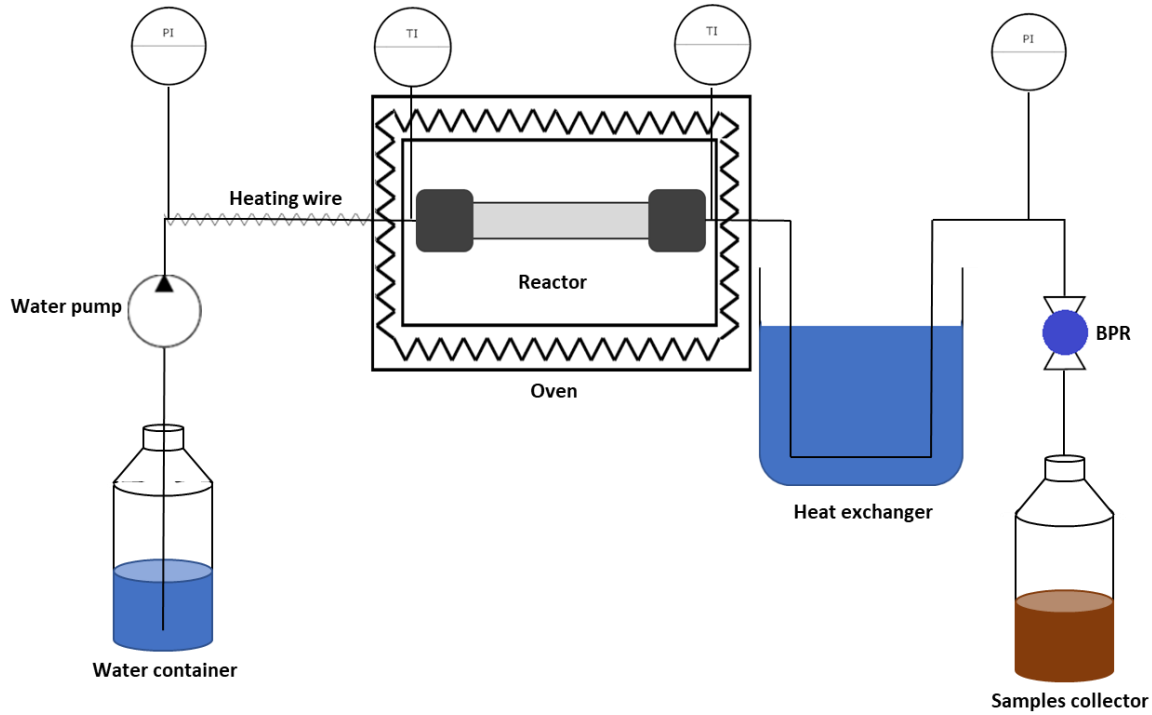

Figure 2.1 – Schematic of the SBW experimental semi-continuous setup. PI is a pressure indicator and TI a temperature indicator. BPR is the back pressure regulator.

For phenolic compounds and hemicellulose recovery, different temperature programs, consisting of ramps and plateaus for given lengths of time, were applied. The water flow rate used was 5-10 mL/min, while pressure was kept constant at 100 bar. Liquors were stored at 4 ºC. Liquors were used to quantify carbohydrates, phenolic compounds, and carbohydrate monomers by high performance liquid chromatography (HPLC). Samples of the liquors were

lyophilized to produce RWGP extracts. The extracts were used for the determination of the yield of extract, as well as for the quantification of ash and protein extracted. The extracts were also submitted to a one-step sulfuric acid hydrolysis for conversion of oligomers to monomers, followed by carbohydrate identification and quantification by HPLC. The residue that remained in the reactor after SBW extraction/hydrolysis was analyzed for protein (elemental analysis), and ash. In the case of assays performed with RWGP, the residue was submitted to Soxhlet extraction to determine lipid content. The defatted residue was then submitted to a two-step acid hydrolysis for quantification of carbohydrate and lignin contents. Enzymatic hydrolysis was also carried out for comparison.

For cellulose hydrolysis, hemicellulose-free residue (SBWR) was submitted to temperatures of 230, 250 and 280 ºC. In this set of experiments, the reactor was heated up to the target temperature and left at that temperature for 30 to 60 min. Pressure (100 bar) and water flow rate (10 mL/min) were kept constant. The liquors were analyzed as described above for quantification of the extract and carbohydrates, as well as sugar degradation products, namely furfural and 5-HMF. The amounts of carbohydrates and of lignin in the solid residue left in the reactor after completion of the assay were also determined.

The extent of extraction/hydrolysis of the process was determined from the amount of material loaded into the reactor and the amount that remained in the reactor after conducting an experiment:

$$Extent\ of\ extraction/hydrolysis, \% = \frac{initial\ weight\ of\ material - final\ weight\ of\ material}{initial\ weight\ of\ material} \times 100 \quad (2.1)$$

The yield of extract expresses the amount of material that was recovered in each assay, out of that which was removed from the starting material placed in the reactor. It was calculated by lyophilizing portions of the total amount of liquor collected in each sample, and correcting for the total volume of each sample, so as to obtain the total amount of extract collected throughout the assay:

$$Yield\ of\ extract, \% = \frac{weight\ of\ extract}{initial\ weight\ of\ material} \times 100 \quad (2.2)$$

In addition to experiments performed with RWGP, several assays were carried out using def-RWGP. However, all the data reported, whether obtained with RWGP or with def-RWGP, are

given per 100 g of RWGP, i.e. the mass balance closes upon addition of the amount of lipids extracted.

The delignification extent was determined from the total amount of lignin present in SBWR and the amount of lignin in the solid residue after SBW or organosolv treatment, as shown in equation 2.3.

$$Delignification, \% = \frac{initial\ amount\ of\ lignin - final\ amount\ of\ lignin}{initial\ amount\ of\ lignin} \times 100 \quad (2.3)$$

The cellulose removal was determined from the total amount of cellulose present in SBWR and the amount of cellulose in the solid residue after SBW treatment, as shown in equation 2.4.

$$Cellulose\ removal, \% = \frac{initial\ amount\ of\ cellulose - final\ amount\ of\ cellulose}{initial\ amount\ of\ cellulose} \times 100 \quad (2.4)$$

2.2.4 SBW batch reactor and experimental conditions

Batch reactions for the hydrolysis of cellulose were performed in a Parr high pressure reactor (model 4547). Briefly, the apparatus consists in a 1.2 L stainless-steel vessel equipped with a magnetic stirrer. The temperature inside the reactor was maintained by a heating blanket placed around the reactor, and by a cooling coil inside the reactor. Both heating elements are connected to a temperature controller connected to a thermocouple inside the reactor. The reactor was first pressurized to 50 bar using either industrial nitrogen, or carbon dioxide. The final pressure in each experiment was dependent on the temperature and the gas used.

For this set of experiments the solid:liquid ratio (S:L) of 1:10 was maintained, and only the target temperature and gas used were varied. For each experiment, the reactor was filled with 30 g of dried hemicellulose-free material and 300 mL of distilled water. The reactor was heated under moderate stirring up to 200-280 ºC and left 15 min at the target temperature. Assays took 45 to 60 min total. When end time was reached, the reactor was cooled down and depressurized. The mixture was then separated by vacuum filtration, the liquors were stored for analysis, as described in 2.2.3, and the remaining solid was dried at 80 ºC overnight and weighed before analysis. The extent of extraction/hydrolysis, the yield of extract, and the amounts of carbohydrates and furfural recovered were also determined.

2.2.5 Modified Organosolv

For the delignification of the hemicellulose-free material, a modified organosolv method was applied (Gurgel et al., 2016). Using the Parr high pressure reactor, different parameters such as S:L ratio, temperature, and reaction time, were studied. Briefly, depending on the S:L ratio, the reactor was loaded with 3-30 g of material and up to 600 mL of a (50:50, v:v) ethanol:water mixture. The reactor was then pressurized with carbon dioxide up to the pressure of the caron dioxide cylinder at 25 ^{o}C (ca. 60 bar). The reactor was purged to ensure that only liquid carbon dioxide was present. While increasing the temperature, the pressure increased up to 100-200 bar, depending on the applied temperature. The temperature range used was 140-220 ^{o}C, the S:L ratio range 1:10 -1:50, and reaction time (at the target temperature) varied between 30 and 180 min. At the end of each experiment, the reactor was cooled down and depressurized. The liquid mixture was separated through vacuum filtration and the solid was oven dried, as described earlier. The lignin content was analyzed.

2.2.6 Enzymatic hydrolysis

The residue left in the reactor after SBW treatment and hemicellulose removal was submitted to enzymatic hydrolysis in order to assess the digestibility of cellulose, following a protocol adapted from Selig et al. (2008) and Fockink et al. (2020). To 5 mL of 0.1 M sodium citrate buffer (pH 4.8) were added 20 µL of 10 wt.% sodium azide, 150 mg of the residue,50 µL of Celluclast 1.5 L and 10 µL of Cellic CTec2. Distilled water was then added to reach the total volume of 10 mL. The hydrolysis was performed in a shaking incubator at 200 rpm and 50 ^{o}C. After 72 h samples were withdrawn from each of the flasks, filtered with 0.22 µm nylon filters and analyzed by HPLC, as indicated in section 2.2.7. Blanks were run for substrate and enzymes. Experiments were replicated.

2.2.7 Carbohydrates and inhibitors analysis

To quantify the sugar monomers glucose, fructose, galactose, arabinose, mannose and xylose (jointly), fucose, and sucrose in the solutions obtained during biomass characterization, in the liquors obtained directly from SBW experiments or after submitting RWGP extracts to a one-step acid hydrolysis, and after submitting the residue left in the reactor to a two-step acid

hydrolysis or enzymatic hydrolysis, HPLC analysis was performed. Briefly, a Dionex ICS-3000 system, with electrochemical detection, a 4x50 mm Thermo BioLC Dionex AminoTrap pre-column and a 4x250 mm Thermo Dionex CarboPac SA10 column at 40 ºC was used. The eluent was a 1 mM NaOH solution at 1.2 mL/min. Calibration curves between 25 and 250 mg/L were built for the sugars of interest. Samples were analyzed directly or diluted 10 times to be quantified in the calibration curve range.

The total amount of carbohydrates was determined using the phenol-sulfuric acid colorimetric method. To 500 µL of sample were added 1.5 mL of H_2SO_4 and 300 µL of 5% (w/v) phenol solution, which was well stirred and incubated at 90 ºC for 5 min. After that, the mixture was cooled to room temperature in a water bath. The absorbance was measured at 490 nm. A calibration curve was built using D(+)-glucose monohydrate solutions (0.01-0.1 g/L). The results obtained were expressed in g/L glucose equivalent (Masuko et al., 2005).

The concentrations of 5-HMF and of furfural in the liquors were also determined by HPLC analysis using a Waters 600 equipment, with a 300 mm Biorad Aminex 87H HPX column at 30 ºC. The mobile phase was 10 mN sulfuric acid at a flow rate of 0.6 mL/min. The volume of injection was 20 µL and the analytes were measured spectrophotometrically at 280 nm. Standards were prepared in the concentration range 2.5–80 ppm.

2.2.8 Phenolic compounds analysis

The colorimetric Folin-Ciocalteau method was used to quantify the total phenolic content (TPC) of the RWGP liquors, as well as of RWGP. Prior to the analysis of phenolics, 800 µL of sample were mixed with 120 µL of 100% (w/v) trichloroacetic acid to precipitate proteins, to avoid possible interference. The mixture was incubated for 5 min at -20 ºC and 15 min at 4 ºC. After centrifugation (12,000 *g*, 15 min) the precipitate was discarded.

Then, 20 µL of the recovered supernatant or gallic acid standard were added to a test tube, as well as 1.58 mL of distilled water and 100 µL of Folin-Ciocalteau reagent. After stirring, 300 µL of sodium carbonate solution was added and incubated at 40 ºC for 30 min in a block dry bath. Absorbance was measured at 750 nm. TPC was expressed in mg/L gallic acid equivalent (GAE), through the calibration curve (25-750 mg/L). HPLC analysis was performed for the identification of individual phenolic compounds in RWGP, using the same equipment

described in 2.2.7, apart from the photodiode array detector to measure the absorbance at two different wavelengths, 280 and 320 nm. The volume of injection was 25 µL and the flow rate 0.5 mL/min. The column (NOVAPAC C18 WATERS) was kept at 30 ºC. The mobile phase was a mixture of two eluents, A (water and 10 % of methanol) and B (90 % of methanol and water with 2 % (v/v) glacial acetic acid). The program started with 100 % of A for 10 min, gradient 100 % to 85 % A (15 min), 85 % to 50 % A (10 min) and 50 % to 30 % A (15 min). Calibration curves were built for gallic acid, syringic acid, vanillic acid and quercetin.

2.2.9 Antioxidant capacity

The antioxidant capacity of the RWGP extracts and gallic acid was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brás et al., 2015).

The stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol and storing at -20 ºC, for a minimum of 2 h. Then 45 mL of methanol were added to 10 mL of the stock solution and the absorbance of the mixture was measured at 517 nm and adjusted to near 1 by adding more methanol. To 4 mL of the adjusted solution, 150 µL of extract (range of concentrations (50-10,000mg/L) in (50:50, v/v) water:methanol solution), were added. The blank was obtained by adding 150 µL of methanol. The mixtures were stirred and incubated for 40 min, at room temperature. The inhibition of the free radical by each sample was calculated by equation 2.5, where A_{DPPH} is the absorbance of the blank and $A_{extract}$ the absorbance of the sample with extract:

$$\%\ inhibition = \frac{A_{DPPH} - A_{extract}}{A_{DPPH}} \quad (2.5)$$

To express the antioxidant activity of RWGP extracts, the half maximum effective concentration (EC_{50}) was calculated from the inhibition curves obtained. Experiments were replicated.

2.2.10 Antibacterial activity

The Minimum Inhibition Concentration (MIC; lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation) was determined using the broth macrodilution method and the reference bacterial strains *Escherichia coli* K12 DSM498 and *Staphylococcus aureus* NCTC8325. The extracts were

prepared in distilled water and tested in the concentration range 0.3-10 mg/mL. The inoculated LB medium was incubated at 37 ºC for 24 h under constant shaking.

2.2.11 Fourier transform infrared-attenuated total reflection spectroscopy

Fourier transform infrared-attenuated total reflection (FTIR-ATR) analysis was performed to identify the functional groups on lignin and cellulose in the solid residues after organosolv and SBW treatments, to evaluate the efficiency of the treatments. A Perkin Elmer Spectrum Two spectrophotometer was used, and the spectra were acquired in the range 400-4,000 cm^{-1}, with a resolution of 2 cm^{-1} and 16 scans, at room temperature.

2.2.12 Scanning electron microscopy

The surface morphology of solid residues after SBW treatment was analyzed by scanning electron microscopy (SEM) (ThermoScientific Phenom ProX), using an acceleration voltage of 10-15 kV. Samples were placed on an aluminum stub using double-sided carbon tape, and sputter-coated with a thin gold/palladium film in a Quorum Technologies coater, model Q150T ESat. SEM analyses were performed in MicroLab, Instituto Superior Técnico.

2.3 Results and Discussion

2.3.1 Chemical characterization

RWGP had very low water content (4.5 wt.%), as set by lyophilization of the grape pomace supplied. The chemical composition of RWGP on a dry basis is shown in **Table 2.1**. Carbohydrates accounted for ca. 33 %, the amount of soluble sugars being relatively low. The content in lignin was fairly high, consistent with the fact that grape pomace includes seeds which have a comparatively higher amount of lignin than pulp or skins. Protein and lipids are present in significant amounts. Gallic acid was the most abundant phenolic compound identified in RWGP, at 3.9 mg/100 g_{RWGP}. Syringic acid was present at similar levels, while vanillic acid was ca. three times less abundant, and quercetin was detected at levels below 0.5 mg/100 g_{RWGP}.

Table 2.1 - Composition of RWGP on a dry weight basis.

Component	wt.%
Soluble sugars	(6.9 ± 1.0)
Glucose	2.8 ± 0.3
Fructose	3.1 ± 0.3
Fucose	1.0 ± 0.4
Cellulose [a]	15.0 ± 1.1
Hemicellulose	(11.3 ± 0.5)
Xylose	4.1 ± 0.3
Arabinose	2.7 ± 0.0
Galactose	2.2 ± 0.1
Mannose	2.3 ± 0.1
Protein	12.5 ± 1.2
Lipids	10.3 ± 1.0
Acid insoluble lignin	30.3 ± 1.3
Ash	7.0 ± 0.4
Phenolics	4.5 ± 0.2

[a] Measured as glucose after hydrolysis

2.3.2 SBW fractionation of phenolics and hemicellulose rich extracts

As referred earlier, the goal was to study the effect of a set of experimental variables on the fractionation of RWGP. In particular, the objective was to dissolve the less resilient fraction of RWGP to obtain hemicellulose-derived carbohydrates and phenolics, recovering a solid residue containing essentially all the lignin and cellulose available in the original material. Additionally, it was intended to assess the possibility of obtaining differentiated liquors/extracts, richer in carbohydrates or in phenolics.

The full symbols in **Figure 2.2** represent results for experiments performed with RWGP obtained by lyophilizing the feedstock as received from the wine producing company. The full symbols and the asterisks refer to different water flow rates, as indicated in the figure legend. In the case of the full symbols, the full squares depict the temperature program used (right Y-axis): the reactor was heated up to 130 ºC, kept at 130 ºC for 30 min, heated up to 190 ºC, and kept at that temperature for 30 min. The rise in temperature was accompanied by an increase in the

amount of extract collected, represented by the full circles (left Y-axis), which was comparatively more pronounced up to 130 ºC. The trend for a stabilization of the yield of extract is seen also in the yield of carbohydrates. At the end of the 130 ºC plateau, the yield of carbohydrates, represented by the full triangles (left Y-axis), was of ca. 10 g/100 g_{RWGP}. As mentioned earlier, in the production of red wine the grapes are not separated from the grape juice prior to fermentation, which makes the amount of nonstructural carbohydrates in RWGP relatively low. However, the value obtained exceeds the amount of soluble carbohydrates of RWGP. Although 130 ºC is lower than normally reported for the dissolution of hemicellulose (Yedro et al., 2014; Yu et al., 2008), submitting the material to this temperature for a certain length of time appeared to lead to the onset of the depolymerization of the hemicellulose structure. The increase in temperature from 130 ºC to 190 ºC and the change in the water properties that ensued, namely higher ionic product, continued to favor the dissolution of hemicellulose, in this case accompanied by an increase in the amount of carbohydrates recovered, up to ca. 14 g/100 g_{RWGP}. Given that the hemicellulose is present at ca. 11 g/100 g_{RWGP}, a total of 18 g/100 g_{RWGP} of carbohydrates should be accessible, suggesting that a longer assay would be needed to recover the total amount of hemicellulose from RWGP when using this type of temperature program. The extraction curve for phenolic compounds (inset; full diamonds) reveals a similar trend: at the end of the plateau at 130 ºC (ca. 120 min after the start of the assays), the yield of phenolics, though continuing to increase, does so at a lower rate.

The extent of extraction/hydrolysis in the assay represented by the full symbols in **Figure 2.2** was 48.4 g/100 g_{RWGP}. This means that the amount of soluble compounds collected in the assay did not make up for the difference in weight of dry RWGP placed in the reactor and the amount of dry RWGP residue left in the reactor at the end of the experiments. In this case, the difference was of ca. 9 g/100g_{RWGP}, which falls within the 5−10 g/100 $g_{biomass}$ interval that is normally found in our assays. This issue will be readdressed further ahead, by means of a mass balance. Failure to measure correctly the residue that is left in the reactor leads to the calculation of a higher extent of extraction/hydrolysis that is not matched by the amount of extract collected via the liquor exiting the reactor, which includes small amounts of compounds that are soluble at the

temperature of the reactor, but precipitate as the liquor exiting the reactor cools down and are collected as well. Another factor may be the loss of volatile species.

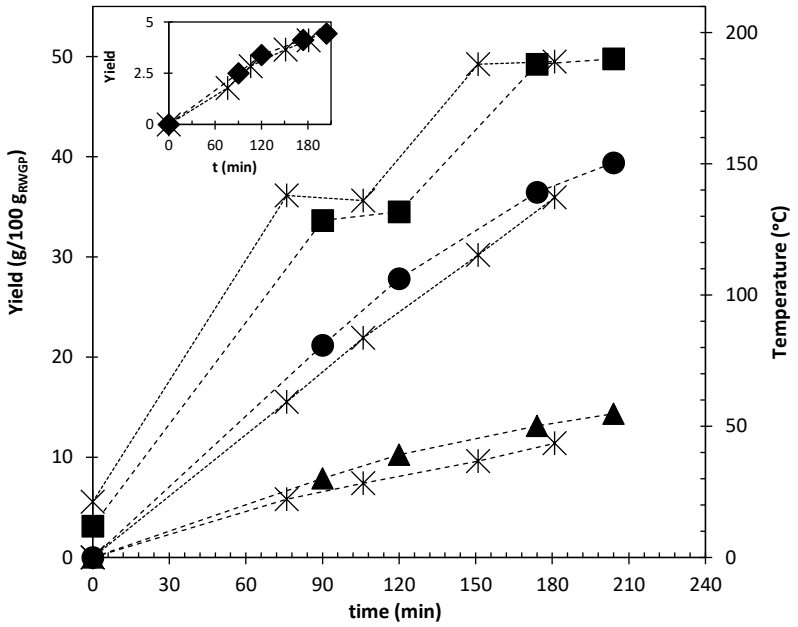

Figure 2.2 - Progress curves for temperature (full squares), yield of extract (full circles), yield of carbohydrates (full triangles), and yield of phenolic compounds (inset; full diamonds) in an assay using a water flow rate of 10 mL/min. The asterisks represent data for an assay performed with a similar temperature program, but a water flow rate of 5mL/min.

The water flow rate is an important process parameter. **Figure 2.2** includes results for an experiment performed with a similar temperature program, but with a lower water flow rate. When taking into account the total amount of water used (i.e. water flow rate multiplied by the duration of the experiment) over the amount of RWGP processed, it is found that reaching the same yield of extract requires a lower water/RWGP ratio when the water flow rate is 5mL/min. Lower water flow rates are equivalent to higher residence times of water in the reactor bed, thus resulting in higher process efficiency. However, at a water flow rate of 10 mL/min, that same yields of extract and of carbohydrates are achieved considerably faster, thereby decreasing advantageously the overall duration of the experiments. The extraction of phenolic compounds, however, seems to have proceeded at the same rate in both cases.

It has been reported that using previously defatted biomass can be advantageous for the recovery of target compounds, due to higher accessibility of the latter (Yedro et al., 2014). We

therefore extracted the lipids from RWGP to obtain def-RWGP for further experiments, as indicated in section 2.2.2.3.

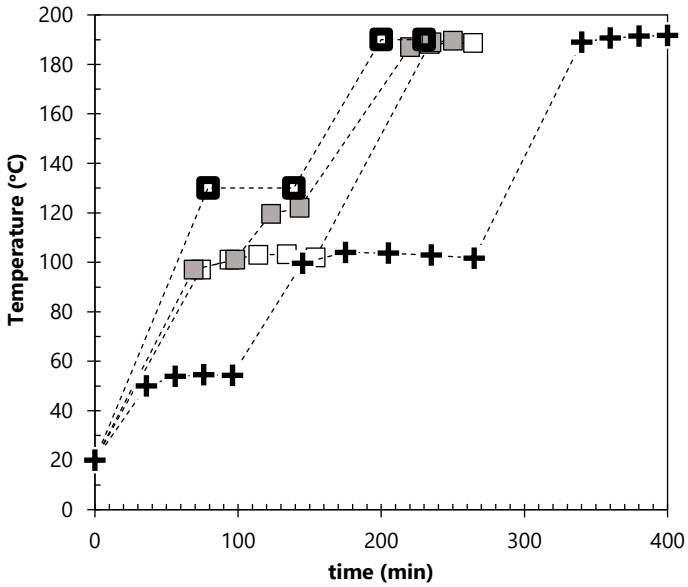

Figure 2.3 – Progress curves for temperature in assays with def-RWGP including plateaus at 130 ºC and 190 ºC (thick border squares), at 100 ºC, 120 ºC and 190 ºC (grey squares), an extended plateau at 100 ºC and another plateau at 190 ºC (empty squares), and three plateaus at 50 ºC, 100 ºC, and 190 ºC (+ symbols). Water flow rate = 10 mL/min. P = 100 bar. Lines are guidelines connecting data points.

To clarify the role of temperature on the extraction of carbohydrates and phenolics, we applied different temperature programs. **Figure 2.3** shows some of the programs that were used in assays performed with def-RWGP, leading to the results shown in **Figure 2.4**. In spite of the slightly different length of the plateau at 130 ºC, the temperature program applied in the assays represented by the symbols with dark border edges was essentially similar to that applied in the assay represented by full symbols in **Figure 2.2**. As shown in **Figure 2.4**, yields of extract and of carbohydrates were also similar (ca. 40g/100 g_{RWGP} and ca. 14 g/100 g_{RWGP}, respectively). The yield of phenolics increased, but together the data obtained do not point clearly towards a positive effect of using previously defatted RWGP.

To look in more detail at the effect of temperature on the transformation of RWGP, experiments were conducted in which the plateau at 130 ºC was replaced by small plateaus at 100 ºC and 120 ºC (grey symbols), or a single, more extended, plateau at 100 ºC (empty symbols). As seen in **Figure 2.4**, the yield of carbohydrates increased in a similar way in the two types of experiments, and stabilized in the value for soluble sugars, only rising when temperature

started ramping up to 190 ºC with release of structural, hemicellulose derived carbohydrates. This behavior was parallel to that of the yields of extract. Irrespective of the temperature program, the amount of carbohydrates recovered tended to a common value of ca. 15 g/100 g_{RWGP}, but did not stabilize, suggesting that a longer assay might improve results. Unlike the yields of extractor of carbohydrates, the yield of phenolic compounds appeared to increase linearly with time. The slightly lower yield of phenolic compounds obtained in the assay including the extended plateau at 100 ºC might be overcome by extending the plateau at 190 ºC.

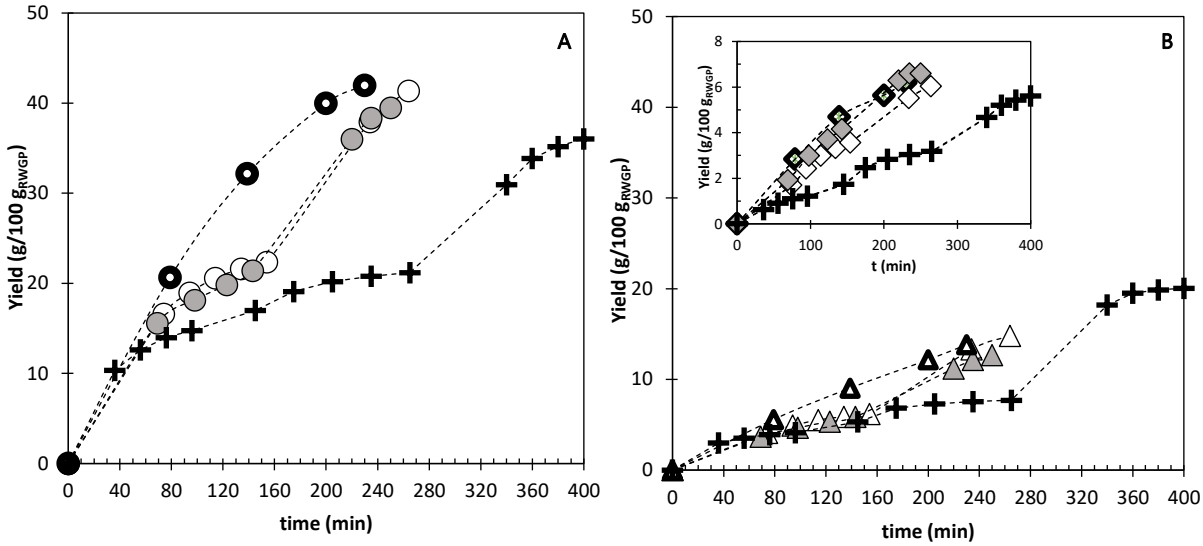

Figure 2.4 - Progress curves for the yield of extract, the yield of carbohydrates, and the yield of phenolic compounds in assays with def-RWGP, performed by applying the temperature programs depicted in **Figure 2.3**, using matching symbols. Water flow rate = 10 mL/min. P = 100 bar. Lines are guidelines connecting data points. A: Yield of extract (circles and + symbols). B: Yield of carbohydrates (triangles and + symbols), and yield of phenolic compounds (inset).

The assays depicted by + symbols in **Figure 2.4** conform to the above findings, first allowing the recovery of the total amount of soluble sugars, and later the recovery of essentially the total amount of hemicellulose derived sugars. In this case the progress curve for phenolics appeared to reflect the three temperature plateaus. The effect of temperature in the assays with def-RWGP can be seen more clearly in **Figure 2.5**, where the yield of carbohydrates is represented as a function of temperature, rather than assay time. Keeping the matrix at a constant temperature had always a positive effect on the release of carbohydrates (datapoints progressing vertically), this effect being more pronounced when structural sugars started to become available. The severity factor, a parameter used to express the intensity of the

hydrothermal treatment as a function of temperature and reaction time (Ruiz et al., 2013), is indeed higher for longer assays at otherwise similar conditions. However, temperature never exceeded 190 ºC, and thus the severity factor was always below 4. Two of the degradation compounds commonly found in lignocellulosic hydrolysates (Yu et al., 2008),namely 5-(hydroxymethyl)furfural, resulting from the degradation of hexoses, and furfural, produced upon degradation of pentoses, were found to be present in the liquors at levels below 0.5 g/L. Most of the carbohydrates recovered from hemicellulose were oligosaccharides (ca. 90 wt.%). Considering that the glucose and fructose monomers measured by HPLC existed as soluble sugars in the starting material, the total amount of the other sugar monomers quantified corresponds to ca. 10 % of the total amount of hemicellulose carbohydrates recovered, as seen in **Figure 2.5**.

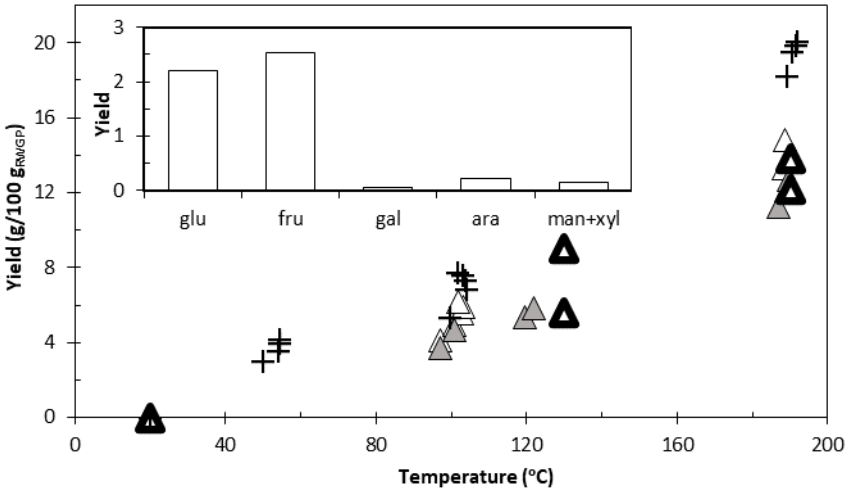

Figure 2.5 - Effect of temperature on the yield of carbohydrates obtained from def-RWGP, as well as average yield of monosaccharides (inset). Symbols match those of **Figure 2.3** and **Figure 2.4**. P = 100 bar. Water flow rate = 10 mL/min.

The antioxidant capacity of the extracts obtained was assayed, leading to the results shown in **Table 2.2** for the assay depicted in full symbols in **Figure 2.2** (RWGP), and the assays depicted in + symbols (def-RWGP). The composition of the extracts collected during an experiment reflects the temperature program used. Most of the ash was recovered in the extracts collected at the lowest temperatures, as evidenced by the ash content of the 25−50 ºC extract, while most of the protein was extracted at the highest temperatures, at conditions bringing about the dissolution of the matrix. In the case of the assays represented by + symbols, the profiles

for carbohydrates and phenolics extraction combined in such a way that extracts collected as temperature ramped up from 50 ºC to 100 ºC and remained at the 100 ºC plateau had a content in phenolics (28.7 wt.%; 28.7 g GAE/100 $g_{extract}$) that was almost twice as high as the other two extracts. This (highest) TPC was accompanied by better (lowest) EC_{50} values. For comparison, the EC_{50} value for gallic acid, the most abundant phenolic compound identified in RWGP and tested at the same conditions as the extract, is included in the table. The antioxidant capacity of the extracts from RWGP compares more favorably with that of gallic acid alone, once the content in phenolics of the extracts is taken into account (lower than 30 wt.%). The EC_{50} values given for the 50−100 ºC and 100−190 ºC extracts are similar to those obtained previously for extracts from white wine GP (Pedras et al., 2017),once similar units are used.

Table 2.2 - Composition (%), antioxidant capacity (EC_{50}), and antibacterial activity (MIC) of extracts obtained in assays represented by + symbols in **Figure 2.4**(three plateaus), and the assay depicted in full symbols in **Figure 2.2**. P = 100 bar. Water flow rate = 10 mL/min.

T (ºC)	Carbohydrates (%)	Protein (%)	Ash (%)	Phenolics (%)	EC_{50} (mg/mg DPPH)	MIC (mg/mL)	
						S. aureus	*E. coli*
25-50 °C	43.4 ± 0.7	5.7 ± 0.1	38.2 ± 1.6	12.7 ±0.4	1.53 ± 0.07	-	-
50-100 °C	52.2 ± 0.9	5.7 ± 0.2	13.4 ± 0.8	28.7 ± 0.8	0.42 ± 0.01	>5	-
100-190 °C	63.6 ± 1.8	20.9 ± 0.9	3.0 ± 0.2	12.5 ± 1.7	0.61 ± 0.01	10	20
25-130 °C	49.4 ± 2.5	10.2 ± 0.3	24.1 ± 0.9	16.3 ± 0.3	0.64 ± 0.01	-	-
130-190 °C	43.3 ± 3.0	29.8 ± 1.2	15.8 ± 0.4	11.1 ± 0.7	0.77 ± 0.02	10	20
Gallic Acid	-	-	-	-	0.035 ± 0.004	-	-

The antibacterial activity of some of the RWGP extracts is also given in **Table 2.2**. This property must depend not only on the amount of phenolic compounds that are present in the extracts, but also on the nature of those compounds, as shown in a study by Cueva et al. (2012), who found that pure gallic acid, the most abundant phenolic compound identified in our RWGP extracts, had high antimicrobial activity against *S. aureus*, unlike other pure phenolics tested. Nevertheless Sanhueza et al. (2014) produced grape pomace extracts from red grape varieties and found a direct correlation between high TPC and high antibacterial activity. In this case, the best extracts were obtained by using extraction with ethyl acetate, which led to a maximum TPC of 11 g GAE/100 $g_{extract}$. These extracts were active against Gram positive and Gram

negative bacteria, namely *S. aureus* and *E. coli*, among others. Fernández-Fernández et al. (2019) have also reported on the antioxidant, antidiabetic, antiobesity, and anti-inflammatory properties of extracts from grape skin, obtained through different extraction methods. Using acid-assisted hydro-alcoholic extraction, the authors obtained extracts with a maximum TPC of 11 g GAE/100 $g_{extract}$. These values and those of the previous authors referred are similar to the TPC values of most of the extracts included in **Table 2.2**. Fernández-Fernández et al. (2019) used more polar solvents than Sanhueza et al. (2014), and provide a more thorough characterization of the grape skin extracts obtained, including carbohydrate content. At most, the latter parameter reached 26 g/100 $g_{extract}$, in the case of the methanol:water:formicacid extraction. As seen in **Table 2.2**, all the extracts listed had higher carbohydrate content, which may counteract the exhibition of antibacterial activity. In fact, MIC values are relatively high, even in the case of the extract with ca. 29 % of phenolics, which did not exhibit a lower MIC value than extracts containing about half the same amount of phenolic compounds. Extracts obtained from white wine GP had lower MIC values when tested against the same bacteria (Pedras et al., 2017). But again, the amount of carbohydrates was lower than in extracts from RWGP, due to the temperature program and sample collection selected.

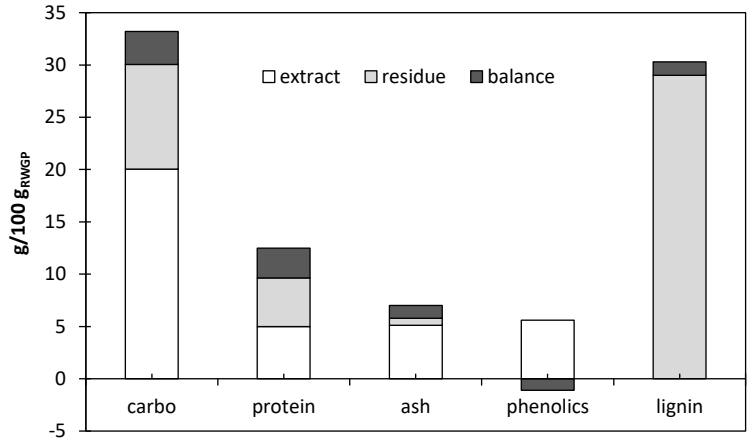

Figure 2.6 - Mass balance for assays represented by the + symbols in **Figure 2.4**. P = 100 bar. Water flow rate = 10 mL/min. Basis: 100 g of dry RWGP (balance closes upon addition of amount of lipids).

The analysis of the extracts obtained, and residual material left in the reactor allow for mass balances, as shown in **Figure 2.6** for assays carried out with def-RWGP (assay depicted in + symbols in **Figure 2.4** as an example). Failure to close the mass balance is usually due to the loss of material, with the exception of phenolic compounds. This type of situation has been

reported before (Aliakbarian et al., 2012), and can be due to the removal of phenolics entrapped in the RWGP structure upon SBW treatment. As indicated earlier, the residue left in the reactor was submitted to a 2-step hydrolysis with sulfuric acid, as well as enzymatic hydrolysis (Rodrigues et al., 2015). Both assays showed that only cellulose remained, as evidenced by the detection of glucose as single sugar monomer. As with cellulose, lignin essentially remained in the residue. When carrying out experiments with RWGP instead of def-RWGP, the residue was also analyzed for lipid content. The data obtained indicated that ca. 87 % of the lipids were left in the residue. Most of the ash content of RWGP was recovered at the lowest assay temperatures. Quantification of protein, on the other hand, revealed that a considerable proportion of protein was undetected in the extract and in the remaining residue.

As illustrated in this study, the outcome of the SBW assays depends on the temperature program selected. When the goal is a practical approach for breaking the hemicellulose structure leaving behind cellulose and lignin, a temperature program consisting of a ramp straight up to a 190 ^{o}C plateau can allow the recovery of most of the soluble and hemicellulose-derived carbohydrates in 2.5 h.

2.3.3 Cellulose and lignin recovery

After phenolics and hemicellulose removal, the solid fraction remaining after SBW treatment (SBWR) was enriched in lignin and cellulose, as seen in figure 2.5. Lignin was the main component, accounting for ca. 80% of the total mass, while cellulose, determined as glucose by acid hydrolysis, represented ca. 12%. The remaining components of SBWR, as shown, are protein and ash.

2.3.3.1 Modified organosolv method for lignin recovery

For lignin recovery an organosolv treatment was studied. The organosolv method produces low molecular weight fragments of lignin with high purity, and a solid rich in cellulose that can be used or converted into glucose by enzymatic saccharification (Michelin et al., 2018). For lignin depolymerization, the cleavage of the α- and β- ether bonds is essential, which is dependent on the pH. The most common organosolv methods use a mineral acid, which in this study was replaced by high pressure carbon dioxide to maintain the process greener. As reported by other authors for the delignification of sugarcane bagasse, the increase in acidity of the medium increases the extent of delignification (Gurgel et al., 2016).

To study the effect of different conditions of the organosolv process on RWGP, different solid:liquid ratios (S:L), reaction times and temperatures were tested. The results are shown in Table 2.3.

Table 2.3 – % delignification of original material (SBWR) and lignin content of the residue remaining in the reactor after organosolv treatment assisted by high pressure CO_2 (standard deviation of 12 %).

Experiment	time (min)	Temperature (ºC)	S:L ratio	Delignification (%)	Lignin content of residue (%)
O1	30	140	1:10	10.2	83.5
O2	30	160	1:10	9.3	82.4
O3	30	180	1:10	6.8	90.8
O4	30	200	1:10	18.9	84.1
O5	30	220	1:10	15.3	87.1
O6	60	160	1:10	18.0	77.0
O7	120	160	1:10	11.0	83.1
O8	180	160	1:10	11.8	90.3
O9	60	200	1:10	16.4	83.2
O10	60	160	1:20	15.4	80.7
O11	60	160	1:50	18.7	81.7

The results show that the process had very low efficiency, a high efficiency meaning a high delignification percentage together with a lignin content (%) of residue much below the ca. 80% in SBWR. In 30 min assays, an increase in temperature to 200 ºC and above almost doubled the % delignification, but was accompanied by the extraction of other components, as reflected in lignin contents of the residue that were similar, or higher, than in the starting material. With the goal to maintain cellulose intact and recover a lignin with high purity, temperature was fixed at 160 ºC, where the loss of sugars was negligible. As seen in **Table 2.3**, the increase in reaction time at that temperature did not improve lignin recovery, and after 180 min of treatment a considerable loss of components other than lignin was observed, the lignin content in the residue increasing to ca. 90%. The increase in S:L likewise did not improve the recovery of lignin while keeping the rest of the solid matrix intact.

A number of representative samples, namely O7, O9 and O11, along with the SBWR fed to the reactor were characterized by FTIR-ATR spectroscopy, spectra being shown in **Figure 2.7**. The spectra show small differences between the untreated material (SBWR) and remaining material after organosolv treatment at different conditions. The wide absorption band at 3300 cm^{-1} is

from the O-H stretch, and the bands at 2970, 2924 and 2840 cm^{-1} are from the stretch of methyl or methylene groups. The band at 2924 cm^{-1} disappeared from the treated material, suggesting that transformations in the solid matrix occurred (Michelin et al., 2018). The bands at 1608, 1515 and 1443 cm^{-1} are characteristic of phenylpropane groups vibrations, C-C vibrations in aromatic rings and C-H vibrations from CH_2 or CH_3, and C-H deformations in aromatic rings. The bands at 1270 and 1230 cm^{-1} are characteristic of guaiacyl and syringyl groups, and aromatic phenyl C-O. These bands, alongside with the one at 777 cm^{-1}, have a lower intensity in the treated samples (Manara et al., 2014). The specific bands of carbohydrates are at 1374, 1162, 1114, 1060 and 897 cm^{-1}. The main difference observed in the intensity of these bands is in sample O9, recovered from the assay at 200 ºC and 60 min length, which suggests that part of cellulose was extracted during the delignification process (Araújo et al., 2019).

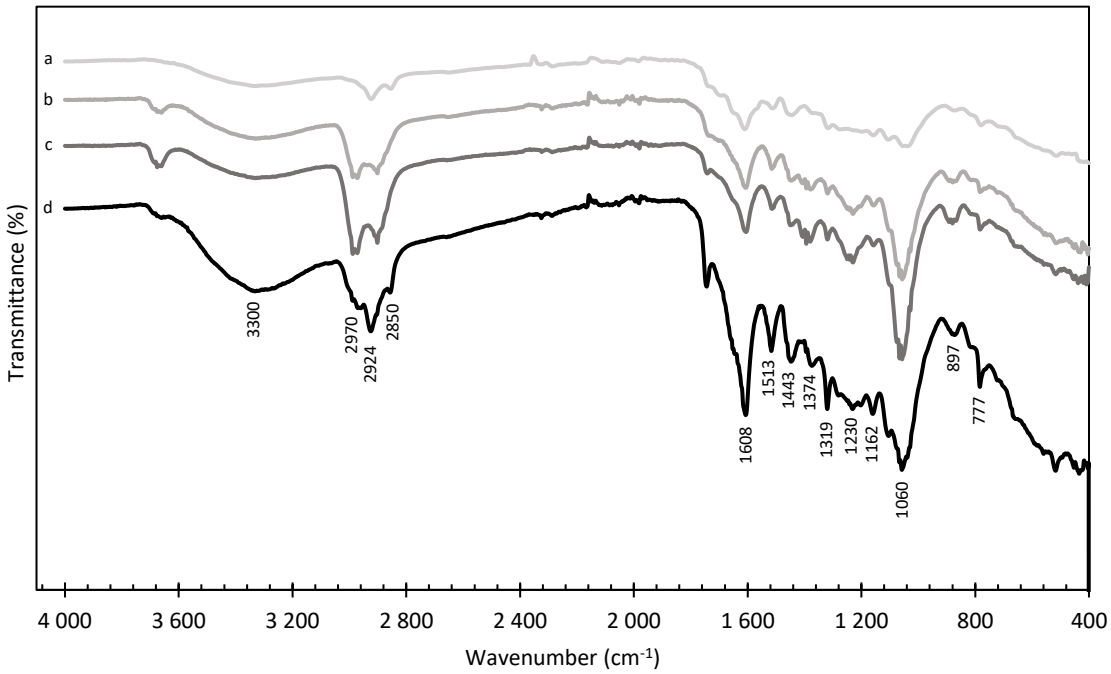

Figure 2.7 - FTIR-ATR spectra of the material before (d) and after (a-c) organosolv treatment. Samples a, b and c correspond to O9, O7 and O11 samples in table 2.3.

The data in table 2.3 and FTIR analysis indicate that this organosolv approach is not suitable for hemicellulose-free RWGP, since unlike other authors report, lignin in not recovered as easily as expected in the liquid fraction (Michelin et al., 2018; Vedoya et al., 2020). Michelin et al. used temperatures between 120 and 160 ºC and recovered over 60% of the lignin from corncob,

after SBW hemicellulose removal. The treated corncob had an initial lignin content of 30%, and ca. 60% of cellulose. During the process the authors obtained a high purity lignin and a good preservation of the cellulose structure (over 90%). Vedoya et al. reported a maximum delignification of 70% for untreated pine biomass, while using the pretreated hemicellulose-free material. The extent of delignification was much lower, reaching values below 40%. In this case the authors say that the effect of CO_2 was only significant above 150 ºC. The pretreatment for hemicellulose removal can influence the efficiency of delignification. In the case presented by those authors the previous hydrothermal process was disadvantageous since lignin became more recalcitrant due to chemical changes. The same effect was reported by Li et al. (2019). These authors say that in a fractionation process of wheat straw components using hydrothermal and organosolv treatments, they observed a clear decrease in delignification from 83.3% to 37.1%, when using the raw material and pretreated material, respectively. The authors found that during hydrothermal treatment a large fraction of lignin migrated from the intracellular layer and spread on the fiber surface, which prevented the dissolution in ethanol. In experiments without pretreatment this phenomenon was not observed. The same effect may have happened in this study, making lignin so difficult to extract at mild conditions without affecting the integrity of cellulose.

The recovery of high purity hemicellulose was an important part in the development of the fractionation process studied in this thesis. Hence, organosolv experiments were only performed after a pretreatment focused on hemicellulose recovery, to avoid obtaining a mixture of lignin and hemicellulose that would need a further separation process.

2.3.3.2 SBW cellulose hydrolysis

In this case, and since the cellulose content of hemicellulose-free material is much lower (12 %) than that of lignin (80 %), the hemicellulose-free material (SBWR) was subjected to a hydrothermal treatment at higher temperatures. The goal was thus changed to the depolymerization of cellulose and glucose recovery, while generating a solid residue rich in lignin, as done by other authors (Yedro et al., 2014). The initial approach was performed in a batch reactor, by testing different temperatures. The effect of CO_2 as an acidifying agent was

also studied with a view to improving the process efficiency (Gurgel et al., 2014), and the results are presented in **Table 2.4**.

Table 2.4 – Extent of hydrolysis, yields of extract and of carbohydrates, % delignification of original material (SBWR) and cellulose removal from the latter (standard deviation of 4 %), in a batch reactor at different temperatures (T). Pressure varied between 50 and 130 bar in experiments with CO_2. The S:L ratio was fixed at 1:10.

Experiment	T (ºC)	Solvent	Extent of hydrolysis (g/100 g_{SBWR})	Yield of extract (g/100 g_{SBWR})	Yield of Carbohydrates (g/100 g_{SBWR})	Delignification (%)	Cellulose removal (%)
B1	250	H_2O	21.5	6.3	0.73	14.1	38.5
B2	280	H_2O	33.8	10.0	0.55	33.0	92.5
B3	200	H_2O:CO_2	10.0	4.0	1.55	0.3	16.7
B4	250	H_2O:CO_2	21.0	6.8	0.86	16.8	40.7

The results show that acidification with CO_2 had no influence on the hydrolysis process, since experiments B1 and B4, performed at otherwise identical conditions, reveal similar extents of hydrolysis, yields of extract and of carbohydrates, and % delignification. In the B3 assay a temperature of 200 ºC was applied, and the hemicellulose-free material fed to the reactor suffered the lowest extent of hydrolysis and % delignification. But although a low yield of extract was obtained, this extract represented the highest recovery of carbohydrates (1.55 in ca. 12). The two highest temperatures tested, 250 and 280 ºC, led to higher % cellulose removal (38.5 and 92.5 %), but this did not translate into a higher recovery of carbohydrates, suggesting that sugar degradation occurred due to the severity of the process. The analysis of 5-HMF and levulinic acid, two degradation products of glucose, confirmed the latter finding, in agreement with Möller et al. (2011). At 250 ºC, 0.06 g/100 g_{SBWR} of 5-HMF was detected, while increasing the temperature to 280 ºC, a slight decrease in 5-HMF was observed (0.03 g/100 g_{SBWR}), accompanied by an increase in the amount of levulinic acid (from 0.30 to 0.83 g/100 g_{SBWR}), indicating that the degradation process was even more severe. Also at 280 ºC, where most of the cellulose was removed, a highly pure lignin was left in the residue. However, this came with a cost since ca. one third of the lignin was lost in the process.

With the aim of increasing glucose recovery, the same approach was studied in a semi-continuous reactor configuration, to avoid sugar degradation. The semi-continuous reactor

worked at similar conditions as presented earlier in sub-section 2.3.2. Water flow rate was fixed at 10 mL/min, and pressure at 100 bar, to avoid water vaporization in the working temperature range. The temperatures of 250 and 280 ºC were initially selected and tested with the new configuration, in which the main difference was that water was passing through SBWR and the extract was collected during the heating and stationary stages. In contrast, the batch mode only allowed recovery of the liquor at the end of the experiment, after cooling down the reactor. The compounds inside the reactor were thus kept for a longer period at high temperatures (higher residence time), making sugar degradation more likely to occur.

The results given in **Table 2.5** show that at 250 and 280 ºC higher yields of carbohydrates were achieved, when compared with the previous experiments. As before, the degradation products were analyzed in the extract, and the amounts detected were much lower than in the batch mode treatments (below 0.01 g/100 g_{SBWR}), although the decrease of the yield of carbohydrates with temperature increase suggest some degradation. The higher yields of carbohydrates obtained could be associated with the recovery of carbohydrates in polymeric form, as cellulose oligomers. Indeed with this reactor configuration, the molecules can be solubilized or dragged and immediately collected, even before complete hydrolysis, since in the SBW hydrolysis process sugar bonds are broken randomly (Cocero et al., 2018). The presence of oligomers is also suggested by the higher yield of extract and extent of hydrolysis compared with the experiments in batch mode. At 280 ºC, the values of those parameters increased from 10 and 33.8 g/100 g_{SBWR} to 27.2 and 59.4 g/100 g_{SBWR}, respectively.

Table 2.5 – Extent of hydrolysis, yields of extract and carbohydrates, % delignification of original material (SBWR) and cellulose removal from the latter (standard deviation of 12 %), in semi-continuous reactor, at different temperatures (T), a pressure of 100 bar and a water flow rate of 10 mL/min. The times given refer to the length of the assay once the target temperature was reached.

Exp.	T (ºC)	time (min)	Extent of hydrolysis (g/100 g_{SBWR})	Yield of extract (g/100 g_{SBWR})	Yield of Carbohydrates (g/100 g_{SBWR})	Delignification (%)	Cellulose removal (%)
S1	**230**	**60**	37.6	26.5	4.7	27.4	37.0
S2	**250**	**30**	42.9	16.1	3.5	31.3	88.8
S3	**280**	**30**	59.4	27.2	3.2	49.0	100.0

Through analysis of the solid residue, it was observed that at 280 ºC all the cellulose was removed, while at 250 ºC ca. 10% of cellulose remained in the original material, which represents much better results than those obtained at similar conditions in the batch reactor (**Table 2.4**). It is reported that, below 280 ºC, cellulose can be dissolved, but only amorphous parts. Our results suggest that most of cellulose present in SBWR was already amorphous, due to the pretreatment suffered or even due to the initial milling of RWGP, which can create amorphous zones that facilitate cellulose dissolution (Cocero et al., 2018).

Regarding lignin, at 280 ºC there was a loss of almost half of the lignin during the process, which can be due to the use a semi-continuous system, since at higher temperatures some lignin fractions can be dissolved and extracted (Cocero et al., 2018). In batch mode, the extract is only recovered after cooling down the system, allowing the repolymerization of lignin, resulting in a lower extent of delignification. In the semi-continuous reactor, there are also higher material losses during handling, as referred in sub-section 2.3.2, which can influence lignin quantification. Nevertheless, the delignification difference is significant.

It is also reported that amorphous cellulose can be easily dissolved at temperatures below 230 ºC and extracted as molecules with high degree of polymerization (Cocero et al., 2018). To overcome the excessive extent of delignification, an experiment at 230 ºC, with a longer rection time at the target temperature (60 min) was performed. The results in **Table 2.5** show that a higher amount of carbohydrates was recovered, and no cellulose polymers were extracted, since the sugars recovered cover the total amount of cellulose removed (4.7 g/100 g_{SBWR}). The % delignification was similar to the one at 250 ºC, bringing no advantage in avoiding lignin losses. Overall, the results suggest that increasing reaction time at 250 ºC could be a possibility to obtain an even more pure lignin fraction, as at 280 ºC. The temperature of 230 ºC was not effective in the treatment of this material.

To support these results, the solid residues recovered from the reactor at the end of the assays were analyzed by FTIR, and compared with SBWR, as shown in **Figure 2.8**.

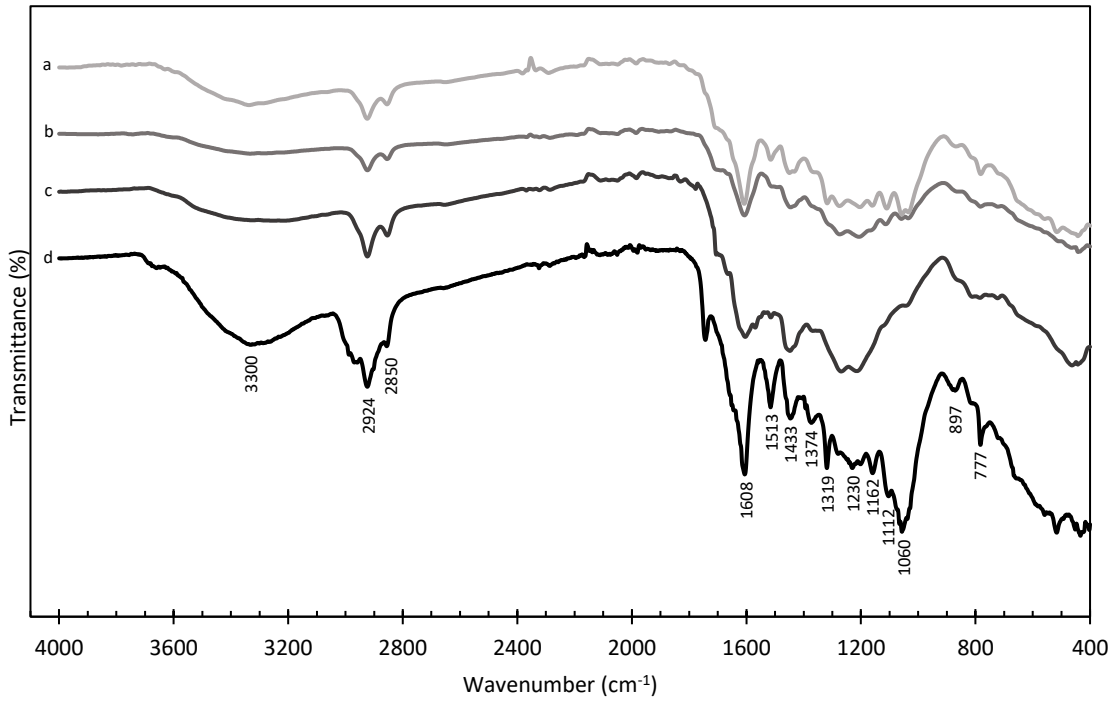

Figure 2.8 – FTIR spectra of the solid residues from experiments S1, S2 and S3 (a-c) and original residue, SBWR (d).

In the FTIR spectra, significant differences between all the three samples can be observed, as well as when comparing with the untreated material. The band at 3300 cm^{-1}, attributed to O-H stretching in cellulose, has much less intensity in the residues from treatments at 250 and 280 ^{o}C. The bands at 1608, 1513 and 1433 cm^{-1}, characteristic of aromatic rings and C-C vibrations, present a decrease in intensity with the temperature increase, suggesting the cleavage of lignin structures at high temperatures (Feng et al., 2019; Manara et al., 2014). On the other hand, the bands at 1270 and 1230 cm^{-1} had an increase in intensity as temperature increased. These bands are characteristic of guaiacyl and syringyl groups, and aromatic phenyl C-O (Manara et al., 2014).

Regarding the specific bands of carbohydrates, a marked decrease in intensity is observed with increasing temperature. The band at 1374 cm^{-1} is from C-H asymmetric deformation, 1319 cm^{-1} from CH_2 wagging, 1162 cm^{-1} from asymmetric C-O-C stretching, 1112 cm^{-1} from C-OH skeletal vibrations, 1060 cm^{-1} from C-O-C pyranose ring skeletal vibration, and 897 cm^{-1} from C-H vibration of β-glycosidic bonds between glucose units (Araújo et al., 2019). These bands are only well observed in the untreated material and in the solid residue after treatment at 230 ^{o}C, where most of cellulose was still present. In the material obtained after treatment at 250 ^{o}C,

bands with very low intensity are observed, confirming that most of cellulose was removed, while at 280 ºC none of the bands characteristic of carbohydrates are observed, since the carbohydrates were effectively removed. The FTIR data agree with the results in Table 2.5. At 280 ºC it was observed 33 % of delignification, FTIR spectra suggesting that the remaining lignin in the solid suffered some modifications.

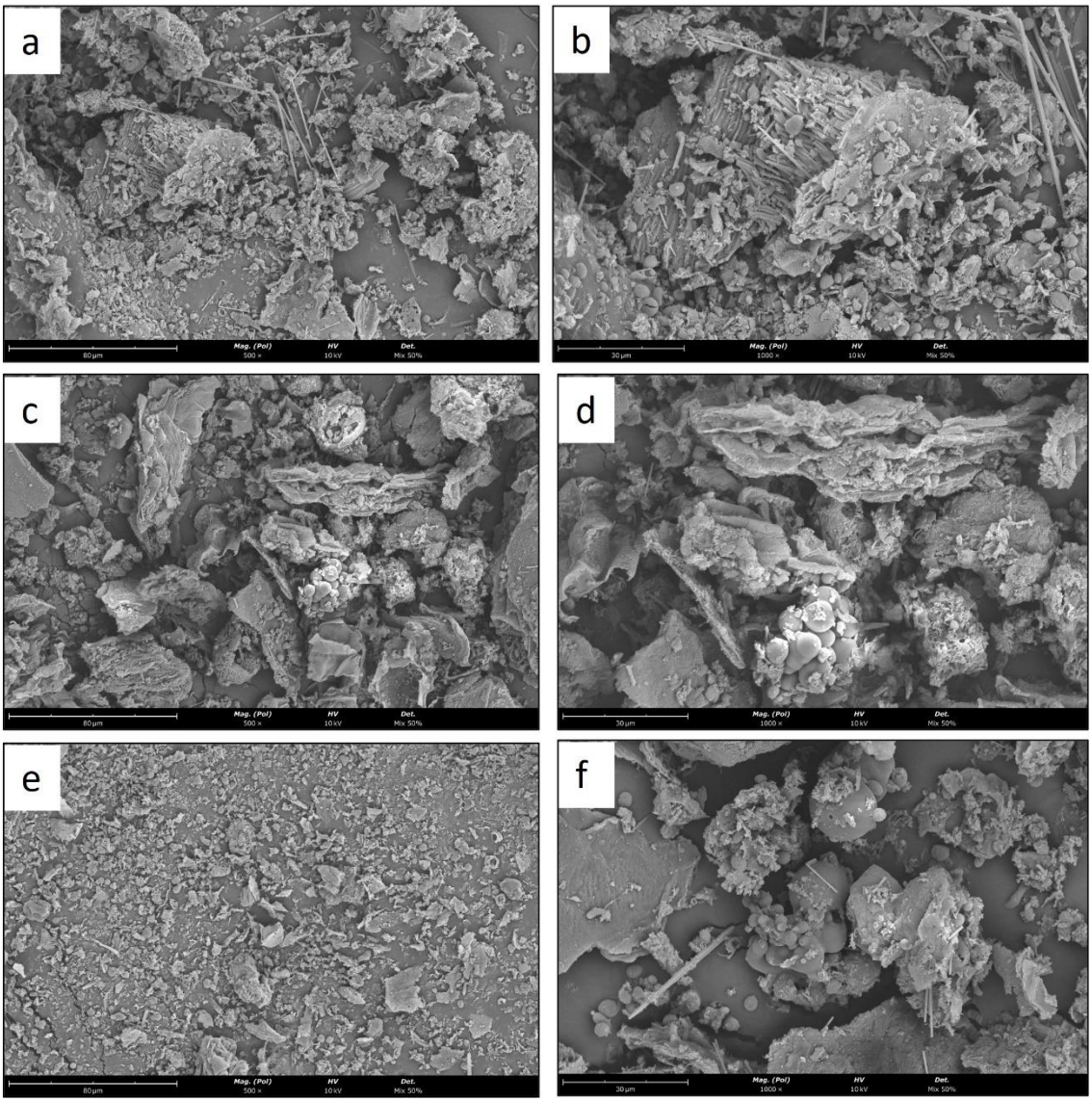

Figure 2.9 - SEM images of the solid residues after SBW treatment at 230 ºC (a and b), 250 ºC (c and d) and 280 ºC (e and f), with magnification of 500x (left) and 1000x (right).

Figure 2.9 shows the SEM images of the three solid residues obtained after the treatments. It is observed in the images that, with the increase of temperature, there is a higher fragmentation of the material, caused by the cleavage of the cellulose and lignin structures. It

was also observed, in the solids treated at 250 and 280 ºC, the presence of carbon spheres with different sizes and shapes, similar to the ones found by other authors at high temperatures (Yedro et al., 2014). Reddy et al. (2015) reported that these spheres are the result of lignin depolymerization and repolymerization with temperature, and that the number of these lignin droplets increase with temperature. Nevertheless, it is also referred that the average size is dependent on the temperature applied. This phenomenon was found at lower temperatures (170-200 ºC), and it is also observed in the images of the solid residue treated at 230 ºC, though in lower amount. The images a and b also show structures that may be fibers of cellulose, since in this experiment most of the cellulose remained in the solid fraction, as demonstrated by FTIR and carbohydrates analysis of the solid residue.

2.4 Conclusions

Red wine grape pomace (RWGP) was first submitted to extraction/hydrolysis with subcritical water (SBW) in a semi-continuous reactor to obtain a phenolics-rich extract, and hemicellulose carbohydrates. By selecting a number of temperature programs with ramps and plateaus at different temperatures up to 190 ºC and for different lengths of time, it was possible to follow in more detail the effect of the SBW treatment, as evidenced by the analysis of both the extracts obtained, and the residue left in the reactor. It was possible to obtain extracts richer in phenolics and less rich in carbohydrates, but further purification would be required (Cocero et al., 2018). And although none of the temperature programs reported here led to an extract with as high a ratio of bioactive compounds to carbohydrates as afforded by some of the reported hydro-alcoholic extraction methods, a more favorable ratio of those two parameters could be achieved with a different temperature program, e.g. extraction of soluble sugars and some phenolics at a lower temperature, followed by extraction of phenolics before hemicellulose deconstruction. Taking into consideration the error associated to the parameters quantified, it was found that mass balances closed for the major components of RWGP with the exception of protein, and that the fractionation of RWGP into hemicellulose, recovered in the liquors, and cellulose and lignin, recovered in the residue, was successfully achieved.

The extraction of lignin from the residue using a modified organosolv method was not successful, with lignin recovery below 20%. The approach to remove cellulose via SBW treatment at higher temperatures (250-280 ºC), and obtain a solid residue rich in lignin, proved to be more suitable for the fractionation of RWGP. The results showed that the semi-continuous reactor was more efficient than the batch reactor for this process. In the semi-continuous reactor, it was possible to obtain a highly pure lignin fraction at 280 ºC, with complete cellulose removal, but loosing half of the initial lignin (49 %). At 250 ºC, a removal of 89% of cellulose was achieved, accompanied by a lower % delignification (31 %), leading to a lignin fraction with a low carbohydrate content. Both experiments failed as regards obtaining the cellulose fraction as soluble sugars, extracted as cellulose polymers or as degradation and volatile compounds, since there is a large gap in the mass balance of the process. At 280 ºC, from the 12 g of carbohydrates removed, only 3.4 g were recovered. In an attempt to recover higher amounts of carbohydrates from cellulose hydrolysis, a lower temperature of 230 ºC was studied. At those conditions, most of the carbohydrates hydrolyzed were recovered, but the process was not efficient, with only 37% of cellulose removal. Since at 280 ºC a high % delignification was found, the temperature of 250 ºC seems to be the most suitable for cellulose removal and recovery of the highest amount of lignin.

3

HEMICELLULOSE APPLICATIONS

The prebiotic activity results in sub-chapter 3.1 were obtained at IBET, under the supervision of Dr. Frédéric Gaspar. The bacterial strains were prepared and maintained by Inês Leonardo.

The results on hemicellulose-based films preparation and characterization in sub-chapter 3.2 were obtained through a collaboration with Dr. Luísa Neves from LAQV - REQUIMTE.

3.1 Hemicellulose-rich extracts prebiotic potential

3.1.1 Introduction

The human intestine contains more than 400 species of bacteria, mostly belonging to the *Bifidobacterium* and *Lactobacillus* genera. Changes in the proportion between these microorganisms and pathogenic bacteria can cause health issues (Lee et al., 2021; Zhang et al., 2018). Prebiotics are non-digestible food ingredients that have a beneficial effect on the host by selectively stimulating the growth of a limited number of beneficial bacterial species in the colon maintaining a healthy intestinal flora balance (Gibson, G. R., Roberfroid, 1995).

Prebiotics should be resistant to acidic and enzymatic digestion and absorption in the upper gastrointestinal tract, fermentable by the intestine's microorganisms and stimulate only the growth of specific bacteria, such as bifidobacteria and lactobacilli (De Jesus Raposo et al., 2016). Different carbohydrates, such as fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and lactulose, can be already used as prebiotics, while others such as xylooligosaccharides (XOS) and arabinoxylans are emerging candidates (Figueroa-González et al., 2019; Mueller et al., 2017). XOS can be suitable candidates since they present the same or better properties when compared with the better-established prebiotics in the market FOS and inulin, which are fructose oligomers with different degrees of polymerization.

Lignocellulosic biomass can be a feedstock for these XOS since it is composed of cellulose, hemicellulose. The hemicellulose is more readily hydrolysable, due to its amorphous and highly branched structure. In red wine grape pomace's (RWGP) hemicellulose, xylose is the most abundant monosaccharide, making it a suitable source of XOS that can be obtained through hydrothermal pretreatment. As studied before with the SBW fractionation process, it is possible to obtain extracts rich in XOS, since the hemicellulose hydrolysis is not complete and most of the carbohydrates are recovered as oligomers.

In this chapter, the *in vitro* prebiotic activity of hemicellulose-rich extracts, obtained in the SBW fractionation process developed before, will be assessed. Three lactobacilli strains will be tested against enteric bacteria, to evaluate the extract potential as a prebiotic.

3.1.2 Materials and Methods

3.1.2.1 Materials

The bacterial strains *Lactobacillus casei* LMG 6904, *Lactobacillus paracasei* LMG 13087, *Lactobacillus rhamnosus* LMG 6400, and the *Escherichia coli* strains LMG 8223, LMG 2092, and ATCC 8739 were selected for the prebiotic activity assays. Inulin from chicory was purchased from Sigma-Aldrich, USA, glucose and agar from Fisher chemical, USA, and Man, Rogosa & Sharpe (MRS) broths were from Condalab, Spain. Tryptic Soy broths (TSB and TSA with agar) were from VWR, USA, and sodium chloride from Panreac, USA.

3.1.2.2 Hemicellulose extract preparation

The hemicellulose extract was obtained from the RWGP treatment as described in 2.2. Several SBW extraction/hydrolysis processes were performed at the optimized conditions, represented with + in **Figure 2.4**, to obtain enough extract to perform the bacterial assays. The extracts recovered between 100 and 190 ºC, rich in hemicellulose oligomers, were used in the assays.

An extract solution with 50 g/L of carbohydrates was prepared to be used in the prebiotic activity assays. The extract was resuspended in distilled water under agitation, after an hour it was centrifuged at 9000 *g* for 15 min at room temperature, and it was then autoclaved at 110 ºC, for 30 min. The same conditions were used to sterilize glucose and inulin.

3.1.2.3 Carbohydrates analysis

Total carbohydrates were quantified by the phenol sulfuric method described in 2.2.7, samples' absorbance was measured at 490 nm and results were expressed in g/L of glucose equivalents through a calibration curve. The monosaccharides were analyzed by HPLC with the equipment described earlier (2.2.7). Calibration curves were used for each compound determination.

3.1.2.4 Phenolic compounds analysis

The phenolic compounds in the extract were quantified with Folin-Ciocalteau's method as described in 2.2.8. Briefly, the samples were diluted, and the proteins were removed by precipitation, before analysis. Then the samples were mixed with folin reagent and sodium

carbonate, and after incubation absorbance was measured at 750 nm. The results were expressed as mg/L of gallic acid equivalents (GAE).

3.1.2.5 Gel permeation chromatography

Gel permeation chromatography (GPC) was used to evaluate the molecular weight of the oligomers present in the extract. Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) was performed in a Smartline, KNAUER, using a Polysep Linear column (Phenomenex, 7.8x300 mm) at 25 ºC. A refractive Index detector was used for detection. 50 µL of sample was injected and 0.1 M of $LiNO_3$ at 0.6 mL/min was used in an isocratic elution. The molecular weights were estimated using a relative calibration with pullulans.

3.1.2.6 Prebiotic activity assay

The different *Lactobacillus* and *E. coli* strains were streaked on MRS agar and TSA plates, respectively, grown at 37 ºC, and stored at 4 ºC, for no more than 2 weeks, until further use. Before each assay, three isolated colonies of each lactobacilli were grown in 5 mL of MRS with glucose, at 37 ºC without agitation, overnight. *E. coli* strains were grown in the same condition but with an orbital agitation of 180 rpm (New Brunswick Scientific™ Innova™ 44 Incubator Shakers). The OD_{600} was then measured for each strain and adjusted to 0.1 using MRS broth. The three normalized *E. coli* strains inocula were then mixed in equal volumes and used together to simulate a diverse set of enteric bacteria in the gut.

The prebiotic assays were preformed according to Zhang et al. (2018) with minor adaptations. The growth media were prepared in 50 mL Erlenmeyer, with a final volume of 10 mL, including MRS broth (2x concentrated) without glucose, carbon source, normalized bacterial inoculum and distilled water were added. Stock solutions of glucose, inulin, and hemicellulose extract at a concentration of 50 g/L of carbohydrates were prepared and used as carbon source solutions. The carbon source concentrations were tested between 2.5 and 10 g/L. To the prepared liquid media 1% (v/v) of the actively growing and normalized bacterial culture was added and then incubated at 37 ºC, with 180 rpm orbital agitation for 24 h. At 0 h and 24 h samples were enumerated using the serial dilution method on MRS agar plates for each probiotic strain and TSA plates for the *E. coli* mix. The results were reported as colony-forming units per milliliter

(CFU/mL) of culture. Each assay, which consisted of following the growth of the three probiotic strains simultaneously with the *E. coli* mix, was performed in triplicate.

3.1.2.7 Prebiotic activity score and index

Prebiotic activity was evaluated through the prebiotic index and prebiotic activity score (Figueroa-González et al., 2019).

The prebiotic index (I_{preb}) is calculated by the following equation:

$$\mathrm{I_{preb}} = \frac{\text{CFU of probiotics in prebiotic}}{\text{CFU of probiotics in glucose}} \tag{3.1}$$

The prebiotic activity score metric also considers the effect of the prebiotic carbohydrate on enteric bacteria growth. As described by Huebner et al. (2007), the prebiotic activity score is calculated by the following equation:

$$\mathrm{A_{preb}} = \frac{(\mathrm{LogP_{24}} - \mathrm{LogP_0})_{\mathrm{prebiotic}}}{(\mathrm{LogP_{24}} - \mathrm{LogP_0})_{\mathrm{glucose}}} - \frac{(\mathrm{LogE_{24}} - \mathrm{LogE_0})_{\mathrm{prebiotic}}}{(\mathrm{LogE_{24}} - \mathrm{LogE_0})_{\mathrm{glucose}}} \tag{3.2}$$

A_{preb} is the prebiotic activity score, LogP are the log of growth in CFU/mL of probiotic bacteria at 24h ($LogP_{24}$) and 0h ($LogP_0$) in prebiotic and glucose. LogE are the log of growth of *E. coli* at 24h ($LogE_{24}$) and 0h ($LogE_0$) in prebiotic and glucose.

3.1.3 Results and Discussion

3.1.3.1 Hemicellulose extract

The hemicellulose-rich extract (HE) obtained from the fractionation of RWGP with SBW was studied as a new prebiotic source. The HE is rich in xylooligosaccharides (XOS) that are known to have prebiotic potential, with the possibility to be incorporated into different food products (Aachary and Prapulla, 2011).

Prior to evaluating the prebiotic activity of the HE, the carbohydrate and phenolic contents of the extract were analyzed. The extract was analyzed both as obtained from the SBW treatment (HE), and after autoclave sterilization (HE-A), which is required to ensure the absence of microbiological contamination during the prebiotic activity assay. The extract stock solution was prepared to have a total carbohydrate concentration of 50 g/L, based on the ca. 60 % of carbohydrates previously presented in **Table 2.2**. From the total carbohydrates, only about 10

% were accounted as monosaccharides and most were recovered as hemicellulose oligomers. Carbohydrates are the main carbon source used for bacterial growth and will affect both enteric and probiotic bacteria. Enteric bacteria are expected to have a better growth in glucose than in the more complex carbohydrates, such as inulin and HE (Zhang et al., 2018). The growth of the probiotic bacteria is expected to be favored by the presence of more complex carbohydrates, when compared with glucose. Figure 3.1 shows the monosaccharides present in the extract. As expected, xylose is the most abundant monosaccharide in the extract, followed by arabinose and glucose. Arabinose, despite not being one of the more abundant in RWGP, is always one of the most abundant in SBW extracts. Arabinose extraction is related to its position in the branches of hemicellulose structure, which makes it easily hydrolysable. The presence of glucose in the extract is explained by the residual amounts present in hemicellulose and by the possible dissolution of a small fraction of cellulose since amorphous cellulose can be more susceptible to hydrolysis at lower temperatures (Cocero et al., 2018).

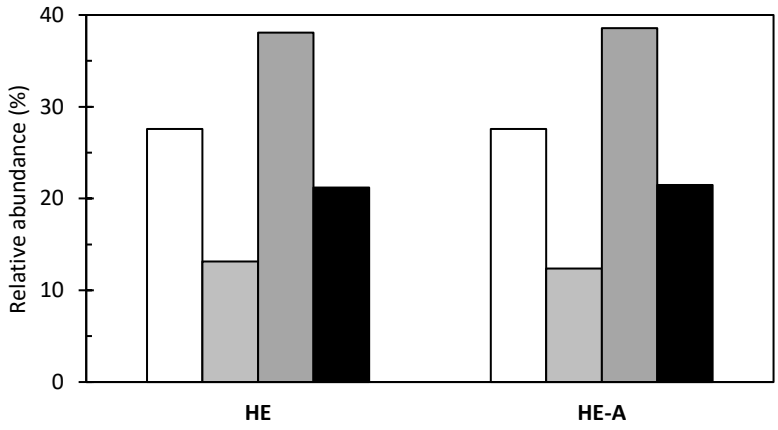

Figure 3.1 - Monosaccharide composition of the hemicellulose extract before (HE) and after (HE-A) autoclave sterilization. From white to black: arabinose, galactose, xylose and glucose.

Gel permeation chromatography analysis was performed to evaluate the molecular weight of the carbohydrate oligomers present in the extract. The results in **Table 3.1** show that the oligosaccharides present in the extract have an average molecular weight of 10.8 kDa. But during heat sterilization using an autoclave, there is evidence that some depolymerization occurred. The most abundant molecular weight (Mn) decreased from 4934 Da to 980 Da. However, the average molecular weight (Mw) was maintained, indicating that the smaller molecules were more affected by the treatment. Another explanation could be the change in

polymer conformation, since it will affect the hydrodynamic volume of the molecule, affecting the results of size exclusion chromatography. These modifications contributed to an increase in polydispersity of almost 6-fold, meaning that the diversity of oligomers may have increased. The decrease of Mn indicates the possible increase of the number of smaller molecules that can more easily be consumed by microorganisms. In all prebiotic activity assays the HE-A was used.

Table 3.1 - Gel permeation chromatography analysis of the hemicellulose extract before (HE) and after (HE-A) autoclave sterilization. Mn is the number-average molecular weight, Mw the weight-average molecular weight, Mp the peak molecular weight, and PD the polydispersity (Mw/Mn). The molecular weights are given in Daltons of pullulan (Da).

Sample (Da)	Mn	Mw	Mp	PD
HE	4934	10854	3581	2.20
HE-A	980	10866	2165	11.09

Apart from the carbohydrates, phenolic compounds were found in a considerable amount, about 11 g/L, in both extract solutions. Since digestive enzymes are not able to degrade these compounds, they can reach the intestine, affecting the intestinal microbiota, due to their antibacterial activity, more specifically against *E. coli* and *S. aureus*, as shown earlier (Chapter 2, **Table 2.2**). Synergetic interactions between phenolics and carbohydrates can also influence the gut microbiota and intestinal health (Lee et al., 2021).

3.1.3.2 Prebiotic activity

To assess the *in vitro* prebiotic activity of the hemicellulose extract (HE-A), three probiotic *Lactobacillus* strains were grown in the presence of different carbohydrates. To be considered a prebiotic, a carbohydrate should be metabolized at least as well as glucose by a probiotic strain. In this study, HE-A was tested alongside inulin and glucose. Inulin is a well-established commercial prebiotic that should behave as a positive control of the assay (Kolida and Gibson, 2007; Park et al., 2022). The three carbohydrates were also tested as carbon sources for a mixture of three *E. coli* strains, which belong to species commonly present in the gut, to simulate the global behavior of enteric bacteria.

Before carrying out any prebiotic activity assay, preliminary tests were performed to determine the optimal parameters to perform the assay in regard to the carbohydrate source and concentration and its effect on the growth of microorganisms (**Table 3.2**). *L. rhamnosus* was initially tested against the enteric mixture using a carbohydrate concentration of 10 g/L to determine the prebiotic activity score as previously described by Huebner et al. (2007).

Table 3.2 – Increase in cell density during 24h, reported as $\log_{CFU/mL}$ for the preliminary bacteria grown in different carbon sources, at 5 and 10 g/L. Enteric mix composed of *E. coli* strains LMG8223, LMG 2091 and ATCC 8739.

concentration (g/L)	carbon source	*L. casei* LMG 6904	*L. rhamnosus* LMG 6400	Enteric mix
10	Glucose	-	3.77	3.22
	Inulin	-	3.42	3.73
	HE-A	-	3.91	-5.48
5	Glucose	2.93	-	3.09
	Inulin	2.99	-	3.89
	HE-A	2.04	-	-5.57

For this concentration, *L. rhamnosus* showed similar growth in the three substrates, while the *enteric* mixture had different behaviors, presenting similar growth in inulin and glucose, while in HE-A no viable cells were found after the 24h of incubation (negative cell density variation between 0 h and 24 h). This result can be explained by the fact that the concentration of extract used is in the range of the minimal inhibition concentration (MIC) determined for *E. coli* in **Table 2.2**. HE-A presented antibacterial activity that did not allow the growth of the enteric mixture. Since the goal of a prebiotic is to have a modulatory effect on the growth of enteric bacteria and not to complete eradicate them, 5 g/L of carbon source was tested and the same inhibitory behavior effect was observed (**Table 3.2**).

By decreasing the concentration to 2.5 g/L, the enteric mixture was able to grow after 24h, as well as the probiotic strains (**Table 3.3**). Between the different lactobacilli strains tested using different carbon sources, different growth patterns can be observed. *L. paracasei* reached a lower cell density when compared with the other two but showed a better performance with the extract than with inulin or glucose.

Table 3.3 - Increase in cell density during 24h, reported as $\log_{CFU/mL}$ for bacteria grown in different carbon sources, at 2.5 g/L. Enteric mix composed of *E. coli* strains LMG8223, LMG 2091 and ATCC 8739.

	***L. paracasei* LMG 13087**	***L. casei* LMG 6904**	***L. rhamnosus* LMG 6400**	**Enteric mix**
Glucose	1.59 ± 0.33	2.50 ± 0.26	3.87 ± 0.16	3.79 ± 0.12
Inulin	1.53 ± 0.21	2.88 ± 0.40	3.70 ± 0.21	4.06 ± 0.17
HE-A	2.04 ± 0.18	2.80 ± 0.05	3.73 ± 0.16	3.56 ± 0.05

The prebiotic effect is usually described in qualitative terms, so the prebiotic index was determined, to evaluate the prebiotic effect of inulin and HE-A (**Figure 3.2**). The prebiotic index obtained with inulin and HE-A as carbon sources were very similar for *L. casei* and *L. rhamnosus*, showing that the substrates do not significantly promote bacterial growth when compared with glucose, since all of them had indexes near 1 and only values above that are associated with a positive prebiotic effect (Figueroa-González et al., 2019). However, for *L. paracasei*, a clear positive effect of HE-A in the growth can be observed, despite reaching a lower cell density (**Table 2.1**).

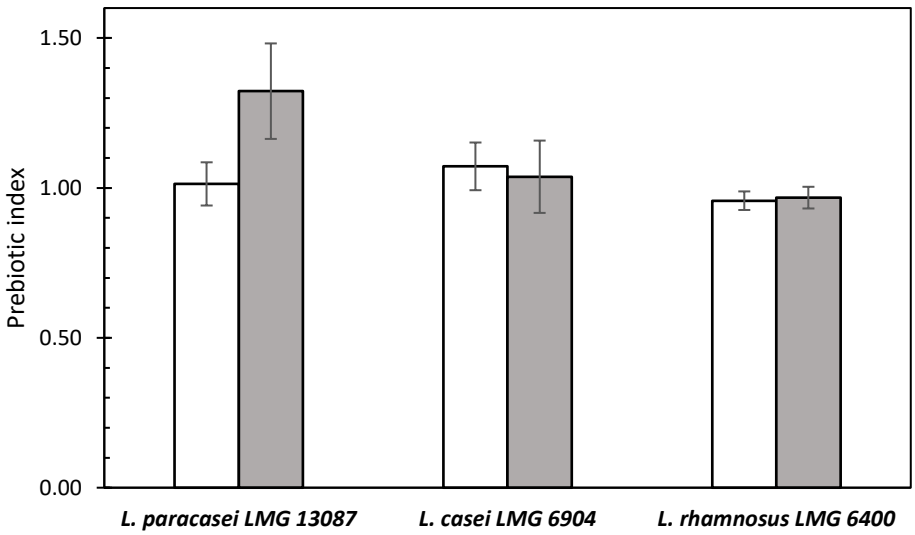

Figure 3.2 - Prebiotic index of inulin (white) and HE-A (grey) for different bacterial strains.

A prebiotic carbohydrate should be easily metabolized by beneficial microorganisms in the gastrointestinal tract, therefore increasing their relative proportion in comparison to other less

beneficial or even deleterious microorganisms. The prebiotic activity scores were then determined to simultaneously include this effect that was not considered by the prebiotic index. For a certain carbohydrate to be considered a prebiotic the score needs to be above 0, and the more effective it is, the higher the score. In Figure 3.3 are shown the prebiotic activity scores for the three lactobacilli studied, with the commercial prebiotic inulin and HE-A as carbon sources.

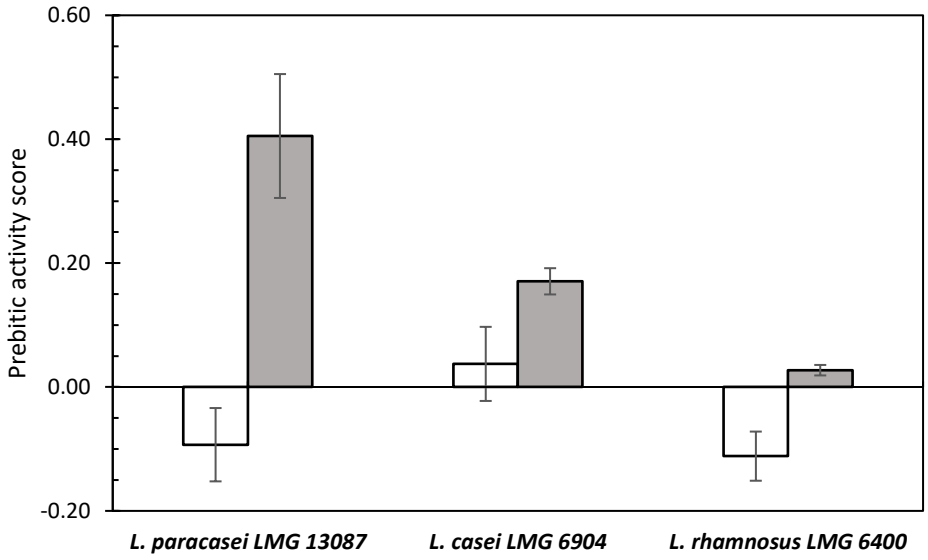

Figure 3.3 - Prebiotic activity score of inulin (white) and HE-A (grey) for different bacterial strains.

The highest prebiotic activity score was obtained for *L. paracasei* grown on HE-A, but a positive effect was observed for the three strains studied with this substrate. The highest score obtained for *L. paracasei* was a combination of both effects, enteric bacteria inhibition and HE-A preference over glucose by the probiotic. Inulin always showed a lower score, presenting only a positive score for *L. casei*. These results can be explained by the growth of the enteric mixture on inulin, also observed by others (Napisah et al., 2022), and the low growth of *L. paracasei* and *L. rhamnosus* when compared with glucose. It has been already reported that different species present different abilities to degrade inulin (Kondepudi et al., 2012; Lee et al., 2021). This will affect the prebiotic activity score. However, for *L. casei* it is still positive, due to its slight preference for inulin over glucose.

The negative effect on *E. coli* growth with HE-A can also reflect the presence of phenolic compounds, which have known antibacterial activity against *E. coli* strains, as demonstrated earlier, and at lower concentrations can be partially inhibiting the growth.

The prebiotic activity scores showed great variability, as expected since there is a high metabolic diversity among *Lactobacillus* strains, which will affect the metabolization of the different prebiotics by the probiotic bacteria (Chang et al., 2022). The differences observed in *L. paracasei* show that there is a preference to metabolize carbohydrates from HE-A when compared with inulin. Inulin and HE-A have very different compositions, since the first is mainly a polymer of fructose molecules, while HE-A has mainly XOS, with some arabinose and galactose from the hemicellulose branches. They not only have different compositions, but also different glycosidic bonds that need specific enzymes to digest (Thuaytong and Anprung, 2011). This variety is also found by some authors between strains of the same species. Figueroa-González et al. (2019) reported very different scores between strains of the species *L. casei* and *L. rhamnosus,* that also showed different scores depending on the prebiotic composition, since the use of each prebiotic by probiotics requires specific hydrolysis and transport systems (Figueroa-González et al., 2019). The same behavior was reported by Huebner et al. for the lactobacilli *L. plantarum* and *L. acidophilus,* where a preference for galactooligosaccharides (GOS) can be also seen but with a wide variety of scores. On the other hand, the *L. paracasei* strain used (1195) showed preference for inulin and other FOS over GOS. (Huebner et al., 2007). This is in line with the obtained results, namely with each strain having a different behavior regarding each prebiotic carbohydrate. However, it is important to note that even with low prebiotic activity scores when compared with some literature (Figueroa-González et al., 2019; Lee et al., 2021), a clear prebiotic effect of HE-A on the three strains tested is observed. It is also observed that *L. paracasei* has clear preference for this substrate, when compared with the other two *lactobacilli*. However, the prebiotic activity scores only show the effect of a particular carbohydrate on the growth of a specific microorganism since this was an *in vitro* study. In gastrointestinal tract some enteric bacteria, as in this study, have metabolic capacity to metabolize potential prebiotic substrates, which will reduce the prebiotic activity and health benefits of the ingestion of these carbohydrates (Napisah et al., 2022). Another issue when studying the prebiotic effect in the microbiome, is the metabolism, because if a

probiotic begins oligosaccharide metabolization via extracellular hydrolysis, the products will be available and a "cross-feed" of other microorganisms can occur (Huebner et al., 2007). So, in a pure culture, a carbohydrate can have a good prebiotic activity score, but when mixed with other microorganisms that are not able to metabolize the oligosaccharides, they still have access to simple sugars. The phenolic compounds effect, however, should be similar, since they can reach the intestine and be effective in controlling enteric bacteria. Nevertheless, the prebiotic activity score is an efficient tool, as proof of concept, to evaluate the effect of a potential prebiotic on specific probiotic bacteria.

3.1.4 Conclusions

The prebiotic index and prebiotic activity score were determined to evaluate the prebiotic potential of the hemicellulose-rich extract (HE) recovered during RWGP fractionation. The two parameters were also determined for inulin, a commercial prebiotic widely used. The highest prebiotic index and prebiotic activity score were obtained for *L. paracasei* grown on hemicellulose-rich extract after autoclave sterilization (HE-A), while the lowest was obtained for *L. rhamnosus* grown on inulin. *L. rhamnosus* did not show a preference for any of the prebiotics, presenting a better growth with glucose. The goal of this study was to evaluate the prebiotic potential of HE-A, and the results show that it was metabolized by all *lactobacilli*, while the *E. coli* mix showed difficulties to grow on this substrate, proving that HE-A has prebiotic activity, as shown by the scores obtained. Since this was an *in vitro* assay using single microorganisms, the behavior in a complex mixture with different microorganisms can be different, but the prebiotic effect is nonetheless clear, mainly for *L. paracasei*.

The next steps in the prebiotic activity study could be to simulate HE-A gastric digestion and evaluate its effect on the intestinal microbiome, thereby confirming the prebiotic activity in conditions similar to the ones found in the human organism. Additionally, the cytotoxicity of the extracts should also be evaluated.

3.2 Hemicellulose-based films obtained from RWGP extracts

3.2.1 Introduction

In the food industry, plastics are the most common material used in packaging since they combine low weight and price with high chemical resistance. Plastic bags are mainly produced from petroleum (Zhao et al., 2020b).

In recent years, new types of packaging materials have been developed. However, the new generation of packaging should have at least the same technical performance as the one currently used, while being degradable and recyclable (Nechita et al., 2021). The new ecological plastics can be used as self-standing packages, edible films, or coatings, acting as barriers to gases, oil, and water. A good barrier material depends on the desired application, with its own requirements as regards gas permeability and light transmission. In the case of food with high content of polyunsaturated lipids, a material with low oxygen permeability is required. When edible films are used to prevent desiccation of fresh fruits and vegetables, the film should have some permeability to carbon dioxide and oxygen, to avoid anaerobic respiration (Mikkonen et al., 2010; Mohamed et al., 2020). Mechanical and thermal properties are other important parameters to take into account when choosing edible or biodegradable films as protective coatings or packaging (Mikkonen et al., 2009; Murrieta-Martínez et al., 2018).

Biopolymers recovered from biomass can be used as alternative source for biodegradable packaging materials. Hemicellulose is currently receiving greater attention due to its biodegradability, biocompatibility, and bioactivity (Nechita et al., 2021). Biodegradable and/or edible films obtained from hemicellulose are being studied to replace the petroleum-based ones, being considered as a sustainable alternative due to their dense macromolecular network with low mobility and high oxygen barrier properties. Hemicellulose-based films are expected to become a new green, membrane material due to their excellent flexibility and barrier properties. The main drawbacks are their mechanical properties. These can be improved by chemical modification, or addition of plasticizers (Huang et al., 2018).

In this part of the chapter, the potential of hemicellulose oligomers obtained from red wine grape pomace (RWGP), through subcritical water (SBW) extraction and hydrolysis, was assessed

with regard to the production of hemicellulose-based films. To improve the quality and strength of the films, the effect of the addition of plasticizers was also studied. Finally, the morphological, chemical, and thermal properties of the films obtained was evaluated.

3.2.2 Materials and Methods

3.2.2.1 Materials

The hemicellulose extract was obtained from RWGP fractionation performed in 2.3.2. As described in 3.1.2.1, the liquor collected between 100 and 190 ºC (table 2.2) was freeze-dried to obtain the extract, stored at -20 ºC in sealed plastic bags to avoid moisture. Sorbitol and glycerol were purchased from Sigma-Aldrich. All the other chemicals were of analytical grade as described earlier.

3.2.2.2 Gel permeation chromatography

Gel permeation chromatography was performed as described in 3.1.2.2, to analyze the molecular weight of the hemicellulose oligomers in the extract. Briefly, Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) was performed using a Polysep Linear column at 25 ºC. A refractive Index detector was used for detection. The mobile phase was 0.1 M of $LiNO_3$ and a calibration curve of pullulans was used to estimate the molecular weights.

3.2.2.3 Films preparation

Films were prepared from the hemicellulose-rich extract, by adding 0.2-0.6 g of the extract to 5 mL of distilled water, and stirring at 50 ºC and 300 rpm for 60 min (Nascimento et al., 2021). Then the mixture was submitted to 4h sonification, poured into a Teflon petri dish, and left to dry overnight at 60 ºC. Experiments were also performed in which a centrifugation step was applied prior to heating, to remove the insoluble fraction of the extract, aiming at an optimization of the film properties. This approach was carried out within a preparation method based on the work of Chadni et al. (2020). Briefly, 1-1.5 g of extract were added to 10 mL of distilled water. The mixture was sonicated. The solid was discarded and the liquid fraction was incubated at 90 ºC and 300 rpm for 10 min. Then the mixture was degassed by sonication for 15 min and poured to dry in a Teflon petri dish, overnight. In some experiments, prior to sonication, a step was included where sorbitol or glycerol were added to the mixture, as

plasticizers, after which the mixture was stirred for 5 min at 60 ºC. Sorbitol was added in an amount corresponding to 40 wt.% of the hemicellulose extract, while glycerol was added at 2% (v/v) of the film-forming solution, as described by other authors (Goksu et al., 2007).

3.2.2.4 Films characterization

3.2.2.4.1 Fourier Transform Infrared-Attenuated Total Reflection Spectroscopy

Fourier Transform Infrared-Attenuated Total Reflection (FTIR-ATR) spectroscopy was performed to determine the film composition (main functional groups), as well as interactions between the different components. As described in 2.2.11, the spectra were acquired in the range 400-4000 cm^{-1} with a resolution of 2 cm^{-1} and 16 scans, at room temperature.

3.2.2.4.2 Thermogravimetric Analysis

Thermogravimetric Analysis (TGA) was performed to evaluate the thermal stability of the films obtained. A TA instrument (Labsys EVO Q50, Setaram) was used with a temperature program between 25 and 500 ºC, with a heat rate of 10 ºC/min. The initial mass used was ca. 16 g. The analysis was performed at Laboratório de Análises, REQUIMTE, FCT-NOVA.

3.2.2.4.3 Scanning Electron Microscopy

The morphology of the films prepared was analyzed by Scanning Electron Microscopy (SEM), as described in 2.2.12. Briefly, small samples were covered with a layer of Gold/Palladium and placed on the SEM device. The samples were submitted to an acceleration voltage of 5-15 kV. The magnifications studied were between 500x and 3000x. For the cross-section analysis, the films were cryo-fractured through immersion in liquid nitrogen before analysis.

3.2.3 Results and Discussion

3.2.3.1 Hemicellulose-based extract

During the RWGP fractionation described in Chapter 2, a hemicellulose-rich extract (HE) was produced (100-190 ºC). This extract was already characterized in terms of composition and bioactivity, as shown in Table 2.2. As referred in 3.1.3.1, the extract presented a high content of carbohydrates and a considerable amount of phenolic compounds. The HPLC analysis of the sugar monomers indicates that most of the carbohydrates were recovered as oligomers, since

very low amounts of monosaccharides were detected - ca. 10% of the total carbohydrates in HE. To evaluate the molecular weight distribution of the carbohydrate oligomers obtained, size exclusion chromatography was performed, as described earlier in 3.1.3.1. Table 3.3 (in 3.1) shows the results of the analysis, and it can be observed that the oligomers present in the extract have an average molecular weight (Mw) of about 10.8 kDa and a polydispersity of 2.2 due to the presence of a large number of low molecular weight molecules (Mn).

3.2.3.2 Hemicellulose-based films preparation and characterization

The hemicellulose extract was used to prepare films via a solvent casting procedure (or solvent evaporation method). The main steps taken in order to obtain viable films are shown in figure 3.4. In the first assays, a procedure used in the production of cellulose films from banana pseudo stem was adopted (Nascimento et al., 2021). The film produced was brittle and cracked during solvent evaporation - figure 3.4 (a). Aiming at the improvement of film strength, a higher amount of extract was used in the procedure (0.6 g instead of 0.2 g). The new film presented slightly improved elasticity, but it was still very brittle, which affected its integrity during the recovery from the petri dish – figure 3.4 (b). Since a significant part of the extract (about 30%) was not water soluble, it was removed by centrifugation and new films were produced only with the soluble fraction. These films were noticeably more resistant and elastic, but still difficult to handle without cracking – figure 3.4 (c).

The addition of plasticizers, namely sorbitol or glycerol, was thus explored. This greatly improved the quality of the films, which were recovered much more easily, without cracking (Chadni et al., 2020). Both plasticizers improved film strength, albeit making them more hygroscopic, since they have the capacity to attract and retain water, as already described by other authors (Mendes et al., 2017). To further improve film properties, the addition of plasticizer was combined with an increase in the amount of extract used, up to 1.5 g. The films obtained using the soluble fraction of 1.5 g of hemicellulose-rich extract and sorbitol or glycerol as plasticizers are shown in figure 3.4 (d) and (e), respectively.

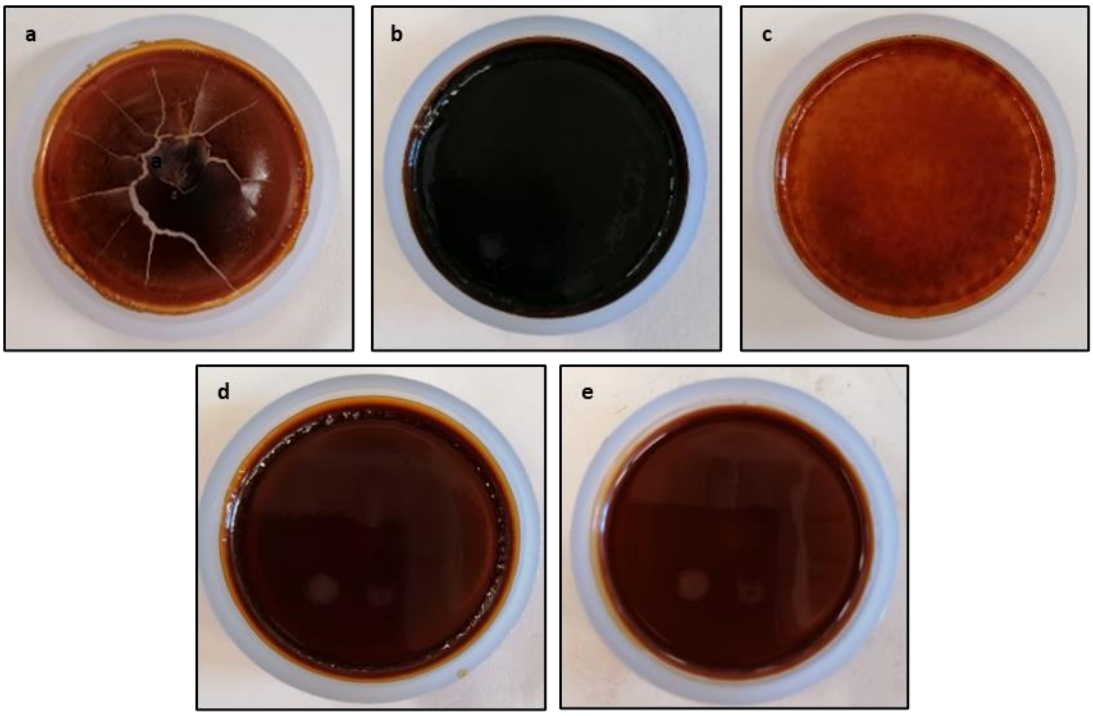

Figure 3.4 – Films based on a hemicellulose-rich extract from RWGP, prepared with the extract as is (a, b), or with the soluble fraction of the extract (c, d, e). In (b), three times as much extract was used than in (a). In (c), no plasticizer was added, while in (d) and in (e), sorbitol and glycerol were used, respectively.

To evaluate the chemical composition of the films, FTIR analysis was performed on films prepared without plasticizer, or with addition of either sorbitol or glycerol, as shown in figure 3.5. The three samples presented similar spectra with some bands with higher intensity due to the presence of the plasticizers, which have similar structure and functional groups as sugars (Mendes et al., 2017).

The broad band at around 3300 cm^{-1} is usually associated with O-H stretching, and can be from the sugars, polyols, and water present in the film. The bands at 2920 and 2884 cm^{-1} are associated with C-H stretching from $-CH_2$ and $-CH_3$, respectively (Egüés et al., 2013; Nechita et al., 2021). Bands at 1634 and 1628 cm^{-1} could be due to the presence of water present in the films, while the band at 1400 cm^{-1} is from C-H and O-H bending associated with the presence of the polyol plasticizers (Mendes et al., 2017). The presence of xylan polymers is confirmed by the characteristic band at 1020 cm^{-1} and at 875 cm^{-1}, characteristic of β-glycosidic bonds between sugar molecules (Egüés et al., 2013). The band at 922 cm^{-1}, which is not present in the

other spectra, is characteristic of glycerol. Additionally, the small band observed at 1534 cm^{-1} can be attributed to the presence of phenolic compounds (Yilmaz-Turan et al., 2020).

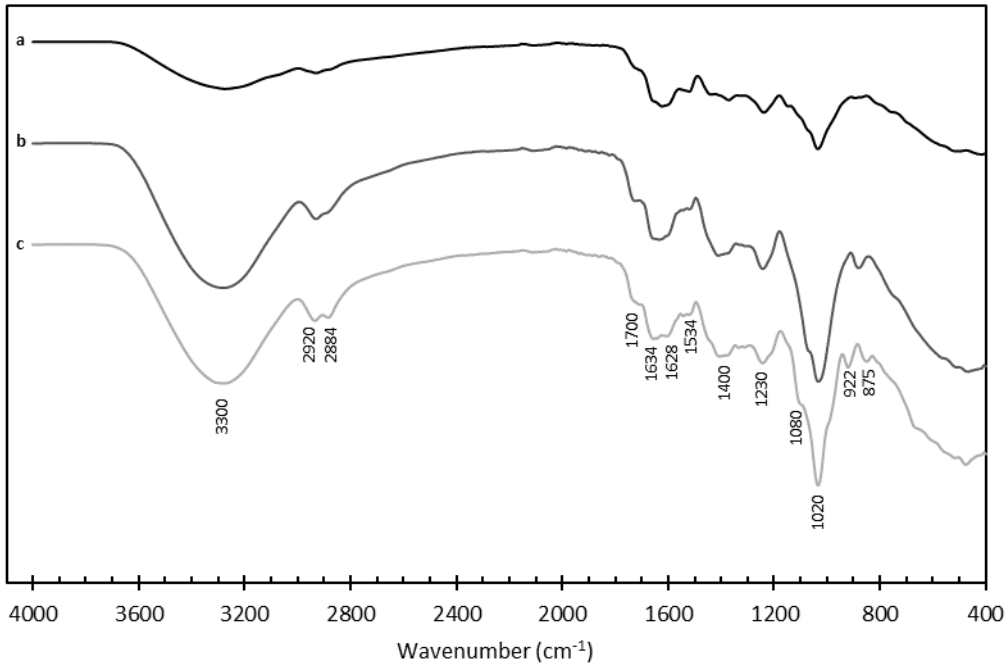

Figure 3.5- FTIR-ATR spectra of the films prepared without plasticizer (a), with sorbitol (b), and with glycerol (c).

These results confirm the presence of the polysaccharides and suggest, as expected, that xylan or xylooligomers (XOS) are the main polymers found in the films. The phenolic compounds can add bioactive properties (antioxidant and antibacterial activity) to the films.

The morphology and thermal behavior of the films with addition of plasticizer were also analyzed. SEM was used for the morphology analysis of the film surface and cross-section, as shown in figure 3.6. The images reveal that both films exhibit a homogeneous surface with a compact and dense structure. However, the one produced with sorbitol exhibits a rougher surface when compared to the one containing glycerol. This could be associated with the higher water vapor transmission rate (Huang et al., 2018). The compactness and high density of the films is the result of a rigid hydrogen-bonded network, promoted by the hydroxyl groups in hemicellulose (Braga and Poletto, 2020), which can be confirmed by the cross-section images that do not reveal any porosity. The ability to establish hydrogen bonds is one of the most important properties of hemicellulose in regard to film formation. The cross-section analysis

also shows a thicker film produced with glycerol, which could be due to the amount water absorbed, since the presence of glycerol increases the hygroscopicity of the films.

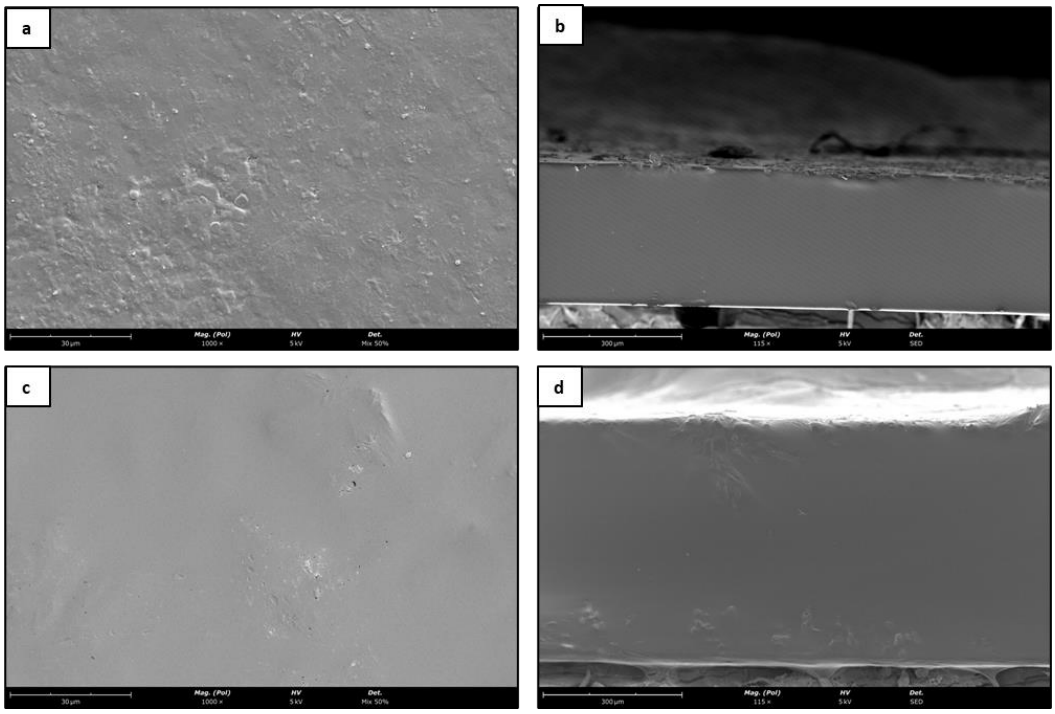

Figure 3.6- SEM images of the films with sorbitol (a) and (b), and with glycerol (c and d). The surface morphology (mag 1000x) is shown in (a) and (c), and the cross section (mag 115x) in (b) and (d).

Thickness is an important parameter to consider for future application since mechanical properties and gas barrier properties are dependent on the film thickness. The increase of thickness can result in lower gas flux and poorer mechanical properties. However the latter can be overcome with the addition of a plasticizer, which can increase film stretchability and water vapor transfer rate (Goksu et al., 2007). The films produced with sorbitol had an average thickness of 350 µm, while the ones with glycerol had an average thickness of 420 µm, which agrees with the SEM images.

To assess thermal behavior, a thermogravimetric analysis was performed, whereby the percentage weight loss of the films was determined as temperature increased. The films prepared with either sorbitol or glycerol present similar thermal degradation curves, as shown in figure 3.7. The films underwent two main weight loss events. The first one, observed for both films between 50 and 160 ^{o}C, is usually associated with the evaporation of water absorbed by the films (Nascimento et al., 2021; Yilmaz-Turan et al., 2020). Regarding the second main event,

small shifts area observed when comparing the film prepared with sorbitol, with the film prepared with glycerol. Using glycerol, the degradation event starts at a temperature around 162 ºC, while using sorbitol the degradation event occurs above 177 ºC. This event is related with the thermal degradation of the plasticizer. The low degradation rate observed may be associated with the complex composition of the films, since they have a mixture of different carbohydrates, phenolic compounds, and even a small amount of proteins. In purified hemicellulose films much clear degradation events, at specific temperatures, are observed, which could overlap in the films prepared in the current work. The main degradation event found by other authors occurs between 180 and 340 ºC, due to the degradation of hemicellulose saccharides to form volatile compounds, such as CO and CO_2, and hydrocarbons of low molecular weight, such as CH_4, C_2H_4 and C_2H_6 (Braga and Poletto, 2020). Another degradation event can occur at 440-500 ºC, which is associated with the degradation of the hydrocarbons formed during hemicellulose degradation (Braga and Poletto, 2020). In the case of the current work, at 500 ºC both films kept around 34% of their initial mass. These values agree with those obtained by other authors for likewise lignin-free films produced by Egüés et al. (2013).

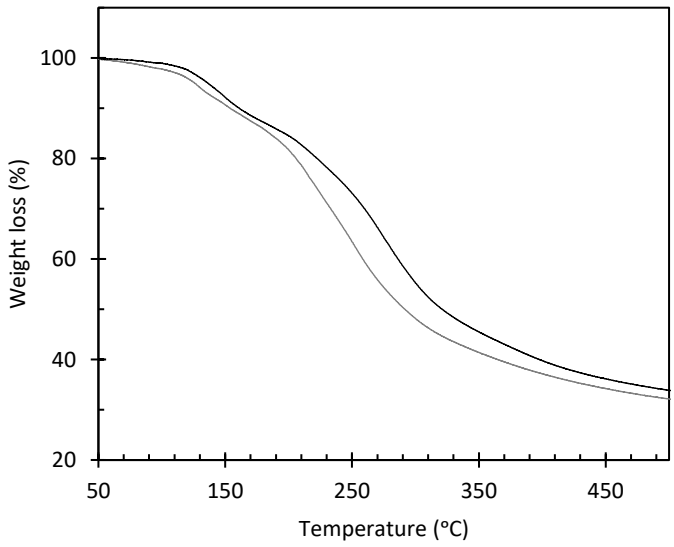

Figure 3.7 - Weight loss as a function of temperature of films prepared with sorbitol (black) or with glycerol (grey).

Overall, the slow degradation rate observed in figure 3.7 should reflect the low purity of the hemicellulose extract used. Moreover the onset of degradation occurs at a lower temperature than for films obtained from purified hemicellulose (Mendes et al., 2017). However, the

presence of phenolic compounds in the films prepared in the current work can be advantageous from the standpoint of antioxidant and antibacterial activities, which precluded purification steps.

Solubility in an aqueous solution is another important property to consider in packaging materials, low water solubility being essential to maintain product integrity. The films produced are completely water soluble, which precludes their use as outer surface barrier. However, high water solubility is desired in the case of food or drug coatings. Additionally, hydrophobic compounds, such as lignin, could be added to the films, to produce materials with a water solubility more similar to that of materials already used for packaging (Braga and Poletto, 2020).

3.2.4 Conclusions

Through the addition of a plasticizer, it was possible to form viable films from a hemicellulose-rich extract produced through the treatment of red wine grape pomace (RWGP) with SBW. Analysis of the films obtained confirmed the presence of xylooligosaccharides as the main carbohydrates. SEM images indicated that the films produced had a compact and dense structure, while thermal analysis showed that the films had a low thermal resistance, the onset of degradation occurring below 200 ºC. The films were also 100% water soluble, which makes them unsuitable as the outermost element of a packaging material. However, they could be included in a multi-layer packaging, or used as a coating or edible film. The addition of lignin, which can also be recovered from RWGP, could make the films less water soluble. The presence of phenolic compounds adds bioactive properties to the films that can be important for some applications.

Improvement of the film properties might also be achieved by using higher chain-length hemicellulose oligomers, as indicated by other authors. E.g. when using steam explosion at 190 ºC to treat lignocellulosic biomass, hemicellulose fragments of over 20 kDa were obtained, while high voltage electric discharges and microwave pretreatments led to Mw values between 47 and 66 kDa (Chadni et al., 2020). Also extraction of arabinoxylan from wheat straw using SBW at 160 ºC led to hemicellulose fragments with an Mw of ca. 80 kDa (Yilmaz-Turan et al., 2020). These results suggest that using a lower temperature (e.g. 130-160 ºC) in the SBW

extraction of hemicellulose from RWGP should allow the recovery of polymers with higher molecular weights. However, since the main goal in Chapter 2 was to remove completely the hemicellulose from the RWGP matrix, the temperature of 190 ºC seemed more suitable to increase the hemicellulose extraction efficiency. Using the same equipment configuration at lower temperatures, and for a longer period, should lead to a reduction of the amount of low molecular weight fragments of hemicellulose, least suitable for film formation. There are also authors who chemically modified hemicellulose fragments, using cross-linking with citric acid, or acetylation of xylan and arabinoxylan chains (Li et al., 2020; Yilmaz-Turan et al., 2020).

Overall, this exploratory work shows the potential to create membranes/films from hemicellulose recovered from RWGP through SBW treatment.

4

WHITE WINE GRAPE POMACE AS CARBON SOURCE FOR THE MICROBIAL PRODUCTION OF LIPIDS AND CAROTENOIDS

The work in this chapter was published as: Pedras, B.M., Gonçalves, C., Figueira, D.R., Simões, P., Gonçalves, P., Paiva, A., Bareiros, S., Salema-Oom, M. White wine grape pomace as a suitable carbon source for lipid and carotenoid production by fructophilic *Rhodorotula babjevae*. Journal of Applied Microbiology. 133 (2022), 656-664.

The search for *FFZ1* and *MtDH1* genes and the discussion of the results was performed by Dr. Carla Gonçalves.

4.1 Introduction

Grapes of *Vitis vinifera* are one of the most abundant crops cultivated worldwide, with available global grape production of almost 78 million tons in 2018, of which nearly 57% were wine grapes (OIV, 2019). Grape pomace (GP) is the solid by-product generated in the wine-making process after pressing the grapes for obtaining the juice. GP is composed of skin pulp and seeds, representing ca. 15 % (w/w) of the total grape input. GP composition depends on the wine-making process. In the case of white wine, GP is separated from the juice before the fermentation onset, leaving this residue with a large amount of soluble sugars (essentially glucose and fructose) that can be easily extracted (Pedras et al., 2017) and used as a carbon source for microbial growth.

Under specific conditions, oleaginous yeasts can accumulate high amounts of intracellular lipids (over 20%, w/w). These lipids, mainly triacylglycerols (TAGs), are typically produced in absence of an essential nutrient (usually nitrogen) while the carbon source is in excess, driving metabolism towards TAG synthesis. Microbial TAGs are similar to plant oil without the need for extensive land fields for cultivation (Maina et al., 2017).

Rhodotorula babjevae is an oleaginous red yeast that in addition to lipid accumulation, synthesizes carotenoids, which are natural lipid-soluble pigments used in the food industry as coloring agents and in the pharmaceutical industry as cosmetics additives (Braunwald et al., 2013; Buzzini et al., 2007b; Schneider et al., 2013). The secretion of glycolipids by *R. babjevae* has also been reported (Garay et al., 2017a). Glycolipids are biosurfactants that can replace the usual detergents derived from petroleum (Garay et al., 2017b). The glycolipids secreted by *R. babjevae* are polyol esters of fatty acids (PEFAs) essentially composed by a polyol head group of D-mannitol or D-arabitol, and an acylated saturated fatty acid, usually C16:0 or C18:0 (Wang et al., 2019). Currently, only a limited number of species belonging to the *Rhodotorula* and *Aureobasidium* genera have been identified to produce polyol lipids (Garay et al., 2018).Since the production costs, such as feedstocks, energy or utilities for complex extractions, are the main barrier for the economic implementation of these products (Manandhar and Shah, 2020), the aim is to explore the soluble sugars from white wine grape pomace (WWGP) as a carbon source for the growth of the non-fastidious yeast *R. babjevae,* and production of lipids and

carotenoids. Because WWGP contains a large amount of easily extracted sugars, unlike RWGP, a biotechnology-based process to obtain different chemicals with market potential can be produced.

4.2 Materials and Methods

4.2.1 Materials

The used WWGP was kindly provided by the Portuguese wine producer Esporão (https://www.esporao.com/), and it was received and stored at -20 ºC, as described in 2.2.1., except the fresh WWGP was oven dried at 60 ºC during 48h instead of freeze drying. Then it was defatted by Soxhlet extraction similar to 2.2.2. Briefly, ca.30 g of WWGP was placed in a 250 mL Soxhlet, and the extraction took place for 6h. In the end, the dry and defatted WWGP was weighted to ensure complete lipid removal. WWGP powder obtained had a particle size of ca. 2 mm and a composition similar to that described (Pedras et al., 2017), with over 45 g soluble carbohydrates per 100 g of dry WWGP. Glucose and fructose accounted for most of the soluble carbohydrates in WWGP and were present in similar amounts.

4.2.2 Sugar extraction from WWGP

For the extraction of soluble sugars, 125 g of defatted WWGP was mixed with 1 L of distilled water and incubated at 30 ºC under magnetic stirring (400 rpm) for 1 h. The mixture was then centrifuged twice (9000 *g*, 10 min, 4 ºC) and the sugar-containing supernatant was temporarily stored at -20 ºC. Additional 125 g of defatted WWGP was used for a second water extraction, but the obtained supernatant was freeze-dried. The freeze-dried pellet was dissolved in the supernatant obtained from the first extraction, resulting in solution S1. The second extraction step had approximately doubled the sugar content of S1 compared to the single extract.

4.2.3 Yeast strain and growth conditions

Rhodotorula babjevae, PYCC 4781, from PYCC (Portuguese Yeast Culture Collection, Caparica, Portugal), was routinely maintained in YPD (yeast extract peptone dextrose) medium. The growth medium used to produce lipids was adapted from Li et al. (2006) with a slight reduction

in sugar and yeast extract. Reference medium, containing commercial sugars, was prepared with 0.1 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 1.5 g/L $MgSO_4 \cdot 7H_2O$, 0.4 g/L KH_2PO_4, 1.91×10^{-6} mM $ZnSO_4$, 1.50 mM $CaCl_2$, 1.22×10^{-4} mM $MnCl_2$, 1.00×10^{-4} mM $CuSO_4$ (pH 6) and ca. 60 g/L of a (1:1) mixture of commercial glucose and fructose, thus giving a carbon to nitrogen ratio of 440:1. The WWGP base-medium contained the same components as the reference medium, but instead of commercial glucose and fructose it contained 75% (v/v) solution S1.

Before each experiment, yeast was pre-cultured on 50 mL of reference medium for 24 h. Yeast growth for lipid production was performed in 1 L shake flasks, with 200 mL of either reference or WWGP-based medium, inoculated with a volume of pre-culture to OD_{640} 0.2. Then, cultures were incubated at 30 ºC and 180 rpm, for 6-8 days, and samples were collected for HPLC analysis, biomass quantification, and pH measurement. At the end, the spent medium was centrifuged at 6000 *g*, for 10 min. The supernatant was stored for glycolipids extraction, and the pellet was washed with distilled water and centrifuged to remove any remaining medium components. Cells were then freeze-dried, to determine cell dry weight (CDW) and stored for intracellular lipids extraction. Duplicate or triplicate experiments were performed.

4.2.4 Intracellular lipids extraction

Intracellular lipids were quantified gravimetrically, using the Folch method with some modifications (Sitepu et al., 2012). A sample of 100 mg of dried cells was placed in a 50 mL falcon tube with 0.5 mm zirconia beads, 3.5 mm glass beads, and 30 mL of Folch solvent (2:1 $CHCl_3$: MeOH, v/v). The mixture was then vortexed five times for 30 s, with resting periods of 30 s in ice between each cycle. After those steps, 6 mL of 0.9% (w/w) NaCl solution were added to improve the separation of cell debris from the extracted hydrophobic compounds. The chloroform-rich phase was recovered and after the complete removal of the solvent, with a nitrogen stream, the lipids were weighed.

4.2.5 Glycolipids extraction

For the extraction of glycolipids (PEFA) (Garay et al., 2017a), four volumes of ethyl acetate were added to the cell-free spent medium in a separating funnel. After agitation, the contents were allowed to rest until complete phase separation. The ethyl acetate phase was recovered and

more four volumes of fresh ethyl acetate were added to the aqueous phase, and the procedure was repeated. At the end, the two ethyl acetate phases were combined, and the solvent was removed using a rotary evaporator. The resulting PEFA extract was stored at -20 ºC.

4.2.6 Acid methanolysis of glycolipids

Acid methanolysis was performed to identify the polyols present in PEFA (Guerfali et al., 2019). Briefly, 2 mL of methanol and 400 µL of 2 M sulfuric acid were added to 20 mg of the dried PEFA extract. The mixture was incubated at 60 ºC for 20 min, after which 2 mL of distilled water were added under agitation. The methanol was then removed with a nitrogen stream, and *n*-hexane was added to solubilize the lipid fraction. The glycosidic fraction of PEFA was recovered from the aqueous phase through phase separation and was neutralized with 1 M NaOH. At the end of the process, samples were filtered through 0.22 µm filters and stored for analysis.

4.2.7 Nitrogen determination

The nitrogen content of the S1 solution was determined through elementary analysis after drying 1 mL of S1 solution at 80 ºC overnight. The analysis was performed at Laboratório de Análises, REQUIMTE, FCT-NOVA.

4.2.8 HPLC analysis of sugars, sugar alcohols and organic acids

The sugars and sugar alcohols were analyzed by HPLC on a Dionex P580 system equipped with an automated sampler injector (Dionex, model ASI-100), an Aminex HPX-87P column with the corresponding microguard cartridge (Bio-rad laboratories), and a differential LKB refractometer, model 2142. Separation was carried out at 80 ºC (Gecko 2000 column heater) using bi-distilled water as mobile phase at a flux rate of 0.6 mL/min, for 35 min. Calibration curves were produced for glucose, fructose, mannitol and arabitol. Tartrate and malate were quantified using the same HPLC equipment after separation on an Aminex HPX-87H column with the corresponding microguard cartridge (Bio-rad laboratories) using 5 mM sulfuric acid as mobile phase at a flux rate of 0.6 mL/min and 65 ºC, followed by UV detection at 210 nm (Dionex diode array detector, model ultimate 3000). The quantification was performed through the calibration curves for malic and tartaric acid.

4.2.9 Phenolic compounds quantification

The total phenolic content of the S1 solution was determined using the colorimetric Folin-Ciocalteau method, as described in 2.2.7. Briefly, samples were diluted, and proteins were removed, before analysis. After incubation of the samples with the Folin reagent and sodium carbonate, absorbance was measured at 750 nm and the results were expressed as mg/L of gallic acid equivalents (GAE), using the respective calibration curve.

4.2.10 Lipid analysis

The fatty acids present in lipid extract obtained in 4.2.4 were identified after derivatization. Briefly, 1.5 mL of 0.5 M methanolic NaOH was added to 25 mg of oil, and incubated for 5 min at 100 °C. Then, 2 mL of BF_3 were added, and again incubated at the same temperature, for 30 min. While the mixture was warm (30 - 40 ^oC), 1 mL of *n*-hexane was added, and the mixture was homogenized before the addition of 5 mL of a NaCl saturated solution. The resulting methyl esters, recovered from the *n*-hexane phase, were analyzed by gas chromatography using an Ultra chromatograph GC (Thermo Scientific), coupled with a flame ionization detector (FID) and a Triplus autosampler (Thermo Scientific). A ZB 5HT Inferno column (Phenomex) was used, with 15 m, 0.30 mm internal diameter, and 0.1 µm film thickness. A split/splitless injector was used, with a split ratio of 1/65, using hydrogen as the carrier gas (1 mL/min). The separation program was 0.5 min at 120 ^oC, 4 ^oC /min ramp up to 230 ^oC, and a 5 min plateau at 230 ^oC. The detector was at 280 ^oC. The internal standard was methyl heptadecanoate, and external standards were methyl oleate, methyl linoleate, methyl stearate, and methyl palmitate, in a range of concentrations between 18 and 0.5 g/L. A mixture of 25 methyl esters was also injected to identify other methyl esters in residual amounts.

4.2.11 Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy

FTIR-ATR analysis was performed to identify the functional groups and the ester bond characteristic of the glycolipids. As in 2.2.10, a Perkin Elmer Spectrum Two spectrometer was used, and the spectra were acquired between 400 and 4000 cm^{-1}, with a resolution of 4 cm^{-1} and 16 scans, at room temperature.

4.2.12 Nuclear Magnetic Resonance (NMR) analysis

NMR analysis of the glycolipids was performed in a Bruker Avance III 400 MHz with a QNP probe, by the NMR Laboratory, REQUIMTE, FCT-NOVA. For the analysis, glycolipids were dissolved in dimethyl sulfoxide-d6, 99.9 atom % D. ^{1}H and ^{13}C spectra were acquired, and HMBC (Heteronuclear multiple bond correlation), HMQC (Heteronuclear multiple quantum coherence), TOCSY (Total correlation spectroscopy), and NOESY (Nuclear Overhauser effect spectroscopy) analyses were performed.

4.2.13 Surface tension

The surface tension of the cell-free culture medium was measured at the beginning and at the end of the experiments to assess the impact of secreted glycolipids (Chebbi et al., 2021). A Sigma 702 (KSV) force tensiometer apparatus was used, with a platinum Du Noüy ring. The density of each solution was also determined. The equipment was calibrated with distilled water.

4.2.14 Search for *FFZ1* and *MtDH1* genes

Local tBLASTn searches were performed with the query sequences of the fructose transporter fz1 from *Starmerella bombicola* (accession number: ALK02043.1), and the mannitol dehydrogenase Mtdh1 sequence from *Yarrowia lipolytica* (XP_503010.1) against the genomes from *R. babjevae* CBS7808 and *R. babjevae* PTZ001 (kindly provided by Marco Coelho, Duke University). The top BLAST hit for each gene was subsequently subject to a BLASTp search against the NCBI non-redundant (nr) database (e-value < e^{-10} and identity > 30 % were used ascutoffs). The presence of the gene of interest was assumed whenever the top hits in the NCBI database corresponded to the query protein orthologues (Ffz1 or Mtdh1).

4.3 Results and discussion

4.3.1 WWGP is a suitable carbon source for *Rhodotorula babjevae*

WWGP is rich in non-structural soluble carbohydrates, which are readily accessible without any prior treatment of the GP matrix, as the one performed before for RWGP. The residue was

previously defatted to increase the extraction efficiency and obtain liquors with higher sugar concentration, essential for the fermentation process. The solution S1 from WWGP contained 43 to 51 g/L glucose and 37 to 44 g/L fructose depending on the batch (soluble carbohydrate extraction yield of ca. 71%), ca. 0.3 g/L of elementary nitrogen, and ca. 1.7 g/L phenolic compounds representing yields of extraction from WWGP of 18% and 50%, respectively (Pedras et al., 2017). Additionally, the WWGP-based medium also contained ca. 4.1 g/L tartrate and ca. 0.3 g/L malate.

Pilot experiments revealed that *R. babjevae* could be cultured in the WWGP-based medium, in which the S1 solution was supplemented with salts and minerals. The pH of the WWGP-base medium was 4 and remained constant during culture probably due to the presence of malate and tartrate or phenolic acids that could buffer the medium. When the pH of the medium was raised to pH 6, the growth of the yeast was not affected. The same was shown for other yeast (Santamauro et al., 2014) that produce higher amounts of lipids at lower pH, so all further work was done with both media at initial pH 4.

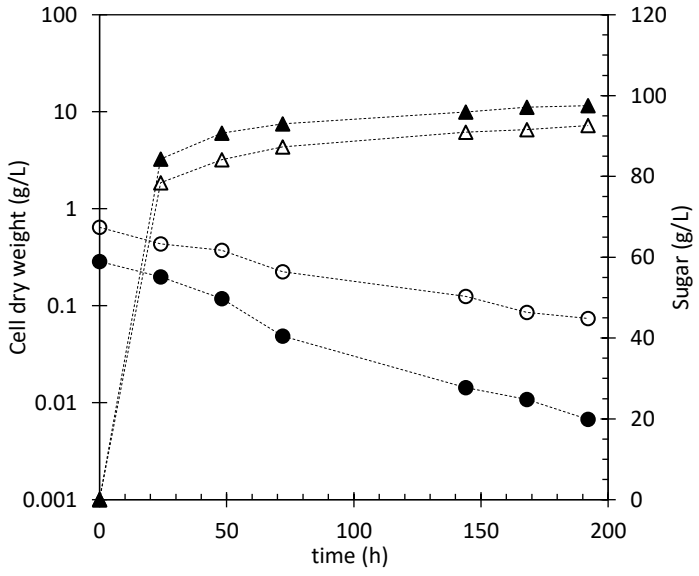

Figure 4.1 - Growth curve and sugar consumption of batch cultures of *R. babjevae* on WWGP medium (closed symbols) and reference medium (open symbols). Samples were periodically taken and cell growth (△, ▲) and total sugar concentration (○, ●) were determined. A representative experiment is shown for each case, but at least three independent assays were performed.

As shown in **Figure 4.1**, *R. babjevae* grew at similar rates in the WWGP-based medium and in the reference medium, although it reached an almost two-fold higher amount of cell dry weight (CDW) when grown in WWGP-based medium. The difference between the content in nitrogen on both media, due to the additional nitrogen provided by WWGP or the content in malate and tartrate might be behind the production of higher amount of biomass, accompanied by a higher sugar consumption, as evidenced in **Figure 4.1**. To explain the higher CDW yield obtained with WWGP-based medium, the reference medium was adjusted to contain similar amounts of nitrogen (added in the form of peptone), malate and tartrate. The results (**Table 4.1**) showed that this adjusted reference medium increased CDW. Surprisingly, tartrate but not malate was consumed in the reference medium, and conversely malate but not tartrate was consumed in the WWGP-based medium.

Table 4.1 - Yield of cell dry weight (CDW) and intracellular lipids, and conversion for assays of different time lengths.

Medium	Harvest time (h)	CDW (g/L)	Intracellular lipids ($g/100g_{DCW}$)	Intracellular lipids (g/L)	Conversion (g_{lipids}/g_{sugar})
Reference medium	144	5.7 ± 0.3	21.5 ± 0.6	1.2	0.14
	192	7.4 ± 0.2	28.7 ± 1.7	2.1	0.08
Adjusted reference medium*	192	9.8 ± 1.2	28.6 ± 0.2	2.8	0.11
WWGP-based medium	144	11.2 ± 1.5	19.4 ± 0.8	2.2	0.07
	192	13.2 ± 1.8	22.5 ± 0.3	3.0	0.06

* Reference medium in which tartrate, malate and N-content were adjusted.

4.3.2 *Rhodotorula babjevae* is a fructophilic yeast

The sugar utilization profile (**Figure 4.2**) unveiled for *R. babjevae* an uncommon behavior manifested in a clear preference for the consumption of fructose over glucose. Fructose preference, designated as fructophily, was evident in both WWGP and the reference media, but while in the WWGP-based medium glucose and fructose were used simultaneously, in the

reference medium glucose was hardly consumed at all, since fructose concentration was still high, maybe associated to the lower growth (**Table 4.1**).

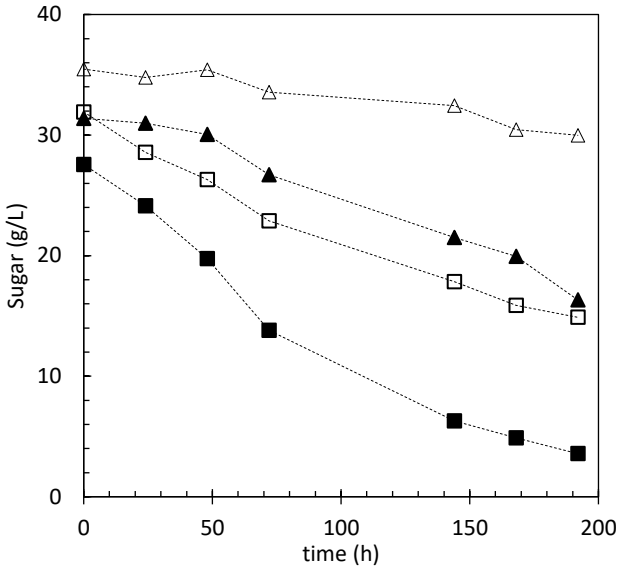

Figure 4.2 - Glucose (△, ▲) and fructose (□, ■) consumption of batch cultures of *R. babjevae* on WWGP-based medium (closed symbols) and reference medium (open symbols), for the same experiment represented in Figure 4.1.

The paralleled consumption of these sugars is not common, as glucose is generally preferred to other sugars present in the medium. Although being a rare feature, fructophily is well-documented for some ascomycetous yeast from the *Wickerhamiella* and *Starmerella* (W/S clade) or *Zygosaccharomyces* genera (Gonçalves et al., 2016), usually associated with niches where glucose and fructose are abundant. However, up to now, it has not been described for *R. babjevae* or other basidiomycetous yeast. In ascomycetous yeasts, the preferential consumption of fructose in the presence of glucose depends on a rather specific high-capacity fructose facilitator, named Ffz1, as well as fructose at high concentrations (Gonçalves et al., 2016), as those observed in WWGP-based or reference medium. Therefore, it was searched for the presence of this gene in the genomes of two *R. babjevae* strains, available at the laboratory, for the presence of the *FFZ1* gene and other relevant genes previously suggested as being associated with this feature. In these two strains we found an *FFZ1* gene encoding the low-affinity, high-capacity fructose transporter intact and possibly functional.

In addition to Ffz1, it was found that both genomes also encoded an NADPH-binding dehydrogenase, with high similarity with members of the NADPH-dependent mannitol

dehydrogenase (Mtdh) proteins in ascomycetous yeasts (44% similarity to YALI0D18964p from *Y. lipolytica*). Mtdh1 in fructophilic yeast, as *S. bombicola*, is responsible for direct reductive dehydrogenation of fructose to mannitol, with concomitant recuperation of NADP+ (Gonçalves et al., 2019). In accordance, *R. babjevae* produced mannitol as a metabolic product, in higher amounts in the WWGP-based medium (1.9 ± 0.0 g/L vs. 0.8 ± 0.1 g/L) in which fructose consumption was more pronounced. Besides mannitol, another polyol, arabitol was detected in spent media.

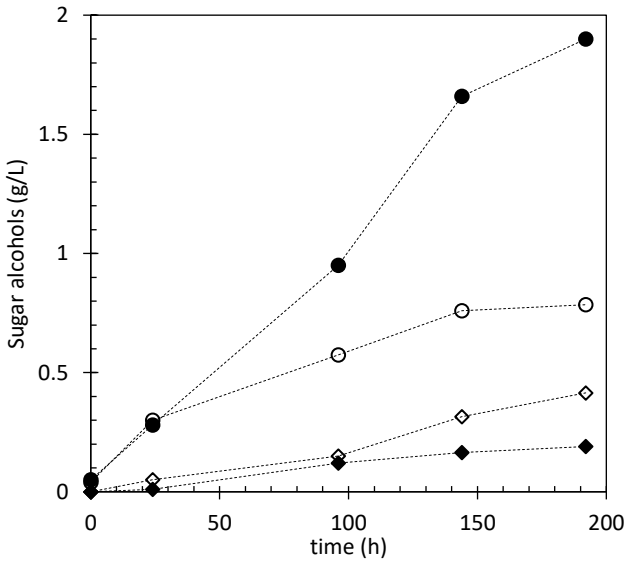

Figure 4.3 - Mannitol (○, ●) and arabitol (◇, ◆) production during batch cultures of *R. babjevae* on WWGP-based medium (closed symbols) and reference medium (open symbols). A representative experiment is shown in each case, but two independent assays were performed.

Fructophily is comprehensively described in *S. bombicola* (Elshafie et al., 2015; Geys et al., 2018; Ribeiro et al., 2013). The preferential consumption of fructose is coupled with the production of lipids and mannitol which results from its direct reduction by mannitol dehydrogenase with concomitant use of NADPH (Gonçalves et al., 2019). In those yeasts, a unique fructose (and mannose) transporter, Ffz1, with high transport capacity and low affinity was found in association with fructophily. The Ffz1-encoding gene appears to have been transferred horizontally from filamentous fungi to a common ancestor of Dikarya (Gonçalves et al., 2016) offering an explanation for its existence in *R. babjevae* and the fructophily disclosed in this work. Likewise, it may justify the efficient use of substrates containing fructose at high concentrations, as in the case of the fructan inulin (Wang et al., 2019) or sucrose (Garay et al.,

2018), described for *Rhodotorula paludigena* or *Aureobasidium pullulans*, respectively, both polyol lipids producers. It is therefore plausible that the molecular underpinnings of fructophily in *R. babjevae* are similar to those previously elucidated in ascomycetous yeasts, but additional experimental work is required to consubstantiate this.

4.3.3 Production of intra- and extracellular lipids

After growth of *R. babjevae* in the WWGP-based medium intracellular TAG accumulation was quantified and found to reach 22.5 % (w/w) (**Table 4.1**), albeit lower than in reference medium (28.7 %, w/w). The increase in CDW observed in modified reference medium did not reduce the lipid accumulation.

The fatty acid profile revealed that oleic acid was the most abundant, followed by palmitic, linoleic, and stearic acids (**Figure 4.4**) as well as residual amounts of myristic and palmitoleic acids in both samples, as found by others (Guerfali et al., 2019; Munch et al., 2015). As expected, *R. babjevae* also accumulated carotenoids, that give the oil the red color, in higher amounts in cells grown on WWGP-based medium (45 ± 1.1 mg/100g_{oil}, corresponding to 10.1 mg/100g_{CDW}) than on reference medium (32 ± 3.2 mg/100g_{oil} or 9.3 mg/100g_{CDW}). Both values fall within the range of total carotenoids production described in the literature, between 6.6 and 11.7 mg/100g_{CDW} (Sperstad et al., 2006).

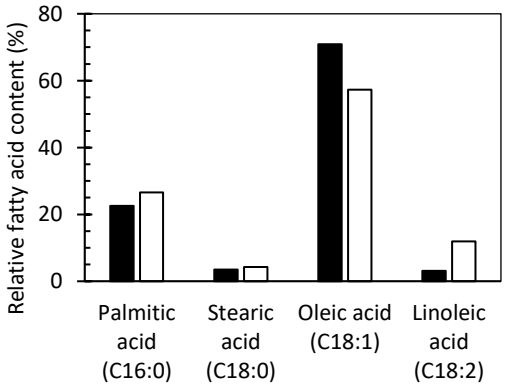

Figure 4.4 - Fatty acid profile of the accumulated intracellular lipids upon growth in WWGP-based medium (black) and reference (white) medium.

A decrease in surface tension was noted, from 56.4 to 30.8 mN/m of the spent WWGP-based medium, which can result from produced extracellular lipid surfactants, but the surface tension

of the reference medium only decreased from 65.5 to 54.2 mN/m. Based on previous reports (Garay et al., 2017a), it was expected that *R. babjevae* secreted glycolipids of the polyol esters of fatty acids (PEFA) type, whose presence would lead to a reduction of surface tension (Singh and Tiwary, 2016). This was confirmed by FTIR-ATR and NMR analysis of the isolated PEFA extracted from spent media. FTIR-ATR spectra showed characteristic bands of the functional groups expected in both polyols and fatty acids, as well as the ester groups that bridge them (**Figure 4.5**). In both spectra, bands at 2978 and 2906 cm^{-1}, corresponding to the elongation of the methyl group (CH_2), and at 1742 cm^{-1}, representing the vibration of the carbonyl group (C=O) of fatty acids, were observed. The distinctive bands of the glycolipids and polyols were found at 3400 cm^{-1}, attributed to the O-H stretch, and at 1025 and 1236 cm^{-1}, attributed to acetyl esters and C-O stretch in sugars and polyols, respectively.

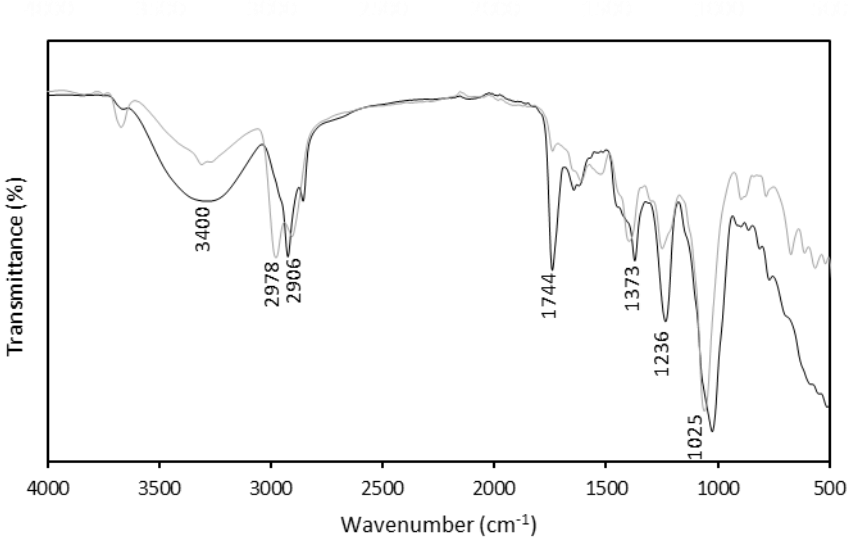

Figure 4.5 - FTIR spectra of the PEFA isolated from WWGP (black) and reference (grey) medium.

To identify the glycosidic fraction, the extracted PEFA were subjected to acid methanolysis to separate the hydrophilic moiety from the fatty acids. Not surprisingly, mannitol and arabitol were detected in the glycosidic fraction, the same polyols already identified as metabolic products. The ester bond bridging the polyol to the lipid was then confirmed by NMR analysis. Analysis of the two-dimensional spectra (HMBC and HMQC) evidenced the interaction between the two protons of the polyol in the ester bond (4.08 and 4.33 ppm) and the carbonyl of the acetyl group (170.9 ppm) of the fatty acid. It was also possible to observe, through TOCSY and HMBC, an interaction between a proton of the hydroxyl group of the polyol (5.2 ppm) and the

carbonyl group (170.9 ppm). The chemical shifts were predicted by Chemdraw software and assigned through ^{1}H and ^{13}C spectra analysis.

A higher amount of PEFA (1.1 ± 0.1 g/L) was found on the ethyl acetate dried extract of spent WWGP-based medium than on reference medium (0.25 ± 0.1 g/L), which agrees with the higher decrease of surface tension measured. Although obtained in relatively low amounts under non-optimized experimental conditions, the lipids produced out of WWGP sugars were identical to those described (Garay et al., 2017a), having a hydrophilic part of predominantly mannitol but also with arabitol, which points to a greater abundance of mannitol PEFAs as expected from extracellular free concentrations of these polyols. The presence of organic and phenolic acids, while making the WWGP-based medium acidic, pH close to 4, did not impact negatively on *R. babjevae* performance. The same was shown for other yeast (Santamauro et al., 2014) that produce higher amounts of lipids at lower pH. Furthermore, Geys et al. (2018) stated a pH of 3.5 to be optimal for sophorolipids production and possibly that is the reason why *R. babjevae* also generated higher amounts of PEFA in WWGP-based medium than in the reference medium.

R. babjevae is known to produce lipids out of a variety of substrates including simple sugars (Guerfali et al., 2019) or cheaper renewable raw materials (Brandenburg et al., 2018). Furthermore, *R. babjevae* is also able to produce PEFA, a specific type of surfactant until now unveiled in just a few species (Garay et al., 2017a). But, contrarily to what is stated for the production of sophorolipids (Ribeiro et al., 2013), lipid production in *R. babjevae* was due to *de novo* lipid biosynthesis, as the WWGP-based medium was prepared from defatted raw material and was thus devoid of any hydrophobic carbon source, as already described for *R. babjevae* strain Y-SL7 and other PEFA producers (Guerfali et al., 2019).

4.4 Conclusions

The main goal was to use the available water-soluble carbohydrates from WWGP as an alternative carbon source for the co-production of high-value products by the non-fastidious yeast *R. babjevae* and create a sustainable process in the context of the circular bioeconomy.

Simple water extraction of WWGP enabled the recovery of solutes, specifically, a reasonable amount of simple sugars. *R. babjevae*, a nutritionally very independent yeast, proliferated on such a minimally processed extract, successfully converting sugars into polyols, TAGs, carotenoids and PEFAs in a manner comparable to a laboratory medium, opening avenues for an alternative form of utilization of by-products from the wine industry.

Moreover, *R. babjevae* metabolized fructose and glucose simultaneously, an unusual feature among microorganisms, undoubtedly convenient for the prompt recovery of these by-products from the wine industry.

Apart from the soluble carbohydrates, WWGP has a similar composition to RWGP, which can be fractionated and valorized, as described in Chapter 2, contributing to a zero-waste policy.

The results demonstrated a fermentation-based possibility for the valorization of WWGP, an agro-industrial residue abundant in the world, using a simple solid-liquid extraction process and a highly versatile yeast that generates a wide range of value-added compounds with great relevance in the pharmaceutical and food market.

5

General conclusions and future perspectives

The work presented in this thesis was focused on the development of processes and applications for the valorization of grape pomace (GP), a by-product of the winemaking industry. Using more sustainable technologies this thesis aimed to contribute to the transition from a linear economy to a circular bioeconomy, in which the use of biomass renewable resources, such as GP, is essential.

The main goal was to fractionate red wine grape pomace (RWGP) into its main structural components, namely cellulose, hemicellulose and lignin, and non-structural components, such as soluble sugars and phenolic compounds. The structural components are usually recovered using hazardous chemicals, such as acids that hydrolyze lignocellulose, and organic solvents, in the case of extraction of phenolic compounds. In this thesis, a cascade process was successfully developed using subcritical water (SBW) for the hydrolysis and extraction of valuable compounds from RWGP. The use of a semi-continuous reactor allowed the recovery of fractions rich in phenolics and hemicellulose, recovered using temperatures up to 100 and 190 ºC, respectively. For the separation of lignin and cellulose, a modified organosolv treatment was first applied to hemicellulose-free material, in a batch reactor, to solubilize lignin and recover cellulose in the solid fraction remaining after the treatment. However, due to the nature of the GP matrix or the chemical modifications suffered in the previous hemicellulose recovery step, the organosolv method was not efficient for lignin recovery, reaching a maximum delignification of 20 %. A shift in approach was thus implemented, whereby the hemicellulose-free material was submitted to a SBW treatment at higher temperatures (230-280 ºC), aiming at the removal of cellulose and preservation of lignin in the solid remaining after treatment. When a batch reactor was used, most of the carbohydrates from cellulose hydrolysis suffered degradation. With the use of a semi-continuous reactor, the degradation of the carbohydrates decreased and at 250 ºC, a small loss of lignin was observed, and most of the cellulose was removed. However, carbohydrate degradation occurred to some extent. To avoid this, lower residence times should be studied. However the SBW apparatus available did not allow the use of higher water flow rates. With the view to future implementation of this alternative greener process, cellulose removal could be integrated with phenolics and hemicellulose fractionation. In a single process, using the SBW semi-continuous reactor and the temperature program

developed, phenolics, hemicellulose carbohydrates, cellulose carbohydrates, could be sequentially obtained, leaving a solid fraction of purified lignin.

The RWGP phenolic-rich extracts exhibited high antioxidant activity, which allow their application in the cosmetic, food or pharmaceutical industries. Some of the extracts presented also antibacterial activity, but this was more pronounced in the extracts with hemicellulose carbohydrates that contained phenolic compounds only extracted after lignocellulose depolymerization.

Additionally, applications for the hemicellulose-rich extracts were studied, both as a source of prebiotics and in the preparation of hemicellulose-based films. The prebiotic activity study showed that the RWGP extract is a potential alternative prebiotic, presenting a good performance. A good prebiotic carbohydrate should be easily metabolized by beneficial microorganisms, as lactobacilli, to increase their relative proportion in comparison to other less beneficial or even deleterious microorganisms. The extract had a positive effect on the different probiotics, in contrast to *E. coli*.

Hemicellulose-based film formation was possible with the addition of sorbitol and glycerol, as plasticizers. Even though the films prepared were considerably thick, they were brittle. The main reason could be the molecular weight of the hemicellulose oligomers (ca. 10 kDa) obtained In the SBW treatment, which was too low. To obtain oligomers with higher molecular weights, lower temperatures could be applied (130-160 ^{o}C) in SBW hydrolysis. Other approaches include chemical modifications of the hemicellulose oligomers, such as cross-linking or acetylation of the chains. Furthermore, a purification step could be added, to separate higher molecular weight oligosaccharides from smaller molecules. The use of longer chain oligomers can improve film formation, while the remaining smaller compounds can be channeled to prebiotics, adding value to both streams.

In the scope of GP valorization, white wine grape pomace (WWGP) was studied as an alternative carbon source for the production of different chemicals with market potential, through a biotechnological approach. WWGP has a high amount of non-structural sugars (glucose and fructose) that can be easily extracted through a simple solid-liquid extraction with water at ambient temperature. The goal was to develop a simple, sustainable and economically

appealing process, using the non-fastidious yeast *Rhodotorula babjevae*. This yeast was able to successfully produce microbial oil and carotenoids from the WWGP sugars, consuming both glucose and fructose, evidencing a fructophilic character that has not been reported before. Apart from the successful production of microbial oil and carotenoids, *R. babjevae* also secreted, simultaneously, polyol esters of fatty acids (PEFAs), a rare type of glycolipids that was just reported by a few authors. Glycolipids are high-value biosurfactants that can replace petroleum-based detergents. The simultaneous production of different valuable products and the use of a minimal processed agro-industrial by-product as a carbon source make this an economically and sustainability interesting process. The next step would be to study the process in a bioreactor on a larger scale, where more parameters, such as oxygen, pH and stirring can be controlled, improving the conditions to achieve higher yields of these high-value products, which will turn the process economically more appealing. In terms of sustainability, the use of organic solvents to extract glycolipids, such as chloroform and ethyl acetate, should be avoided, even more on a larger scale. Supercritical carbon dioxide (sc-CO_2) could be a green alternative solvent for the extraction of these lipids, where the main drawback is that the growth spent media would need to be dried before glycolipids extraction. However, with sc-CO_2 extraction, the final product can be efficiently obtained free of solvent without the need of downstream processes. Furthermore, the solvent can be recycled and used in further extractions.

In the scope of circular bioeconomy, the WWGP residue after sugar extraction can be channeled to the same fractionation process described for RWGP, adding value to the most resilient fractions of lignocellulose.

The main drawback of the implementation of a fractionation process as the one presented is the high variability of lignocellulosic residues composition and chemical structures, which will have an effect on the efficiency of the process. However, the knowledge acquired in the development of the process for RWGP gave important information about the behavior of biomass under specific temperature programs. It should be possible to adapt the fractionation process studied herein to a different material according to its contents of cellulose, hemicellulose, lignin and soluble compounds, as well as to the applications in view.

The main goal of this thesis was the complete valorization of GP. Cellulose can be converted into glucose by SBW or enzymatic hydrolysis and then used by microorganisms for biochemical conversion into added-value products, as the example studied in this thesis. Lignin, as recovered after SBW treatment, can be incorporated in construction materials, as reinforcing or fire-retardant agents. Work on this type of approach began, in collaboration with researchers from the Civil Engineering Department of FCT NOVA.

Overall, the main goals of this thesis were achieved. An approach for the valorization of the wine industry by-product GP was successfully developed, according to green chemistry principles, including a cascade process to obtain differentiated extracts, as well as potential applications for some of them. For the industrial implementation of a biorefinery using this process, scale-up studies need to be done, and a complete evaluation of economic viability needs to be performed.

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